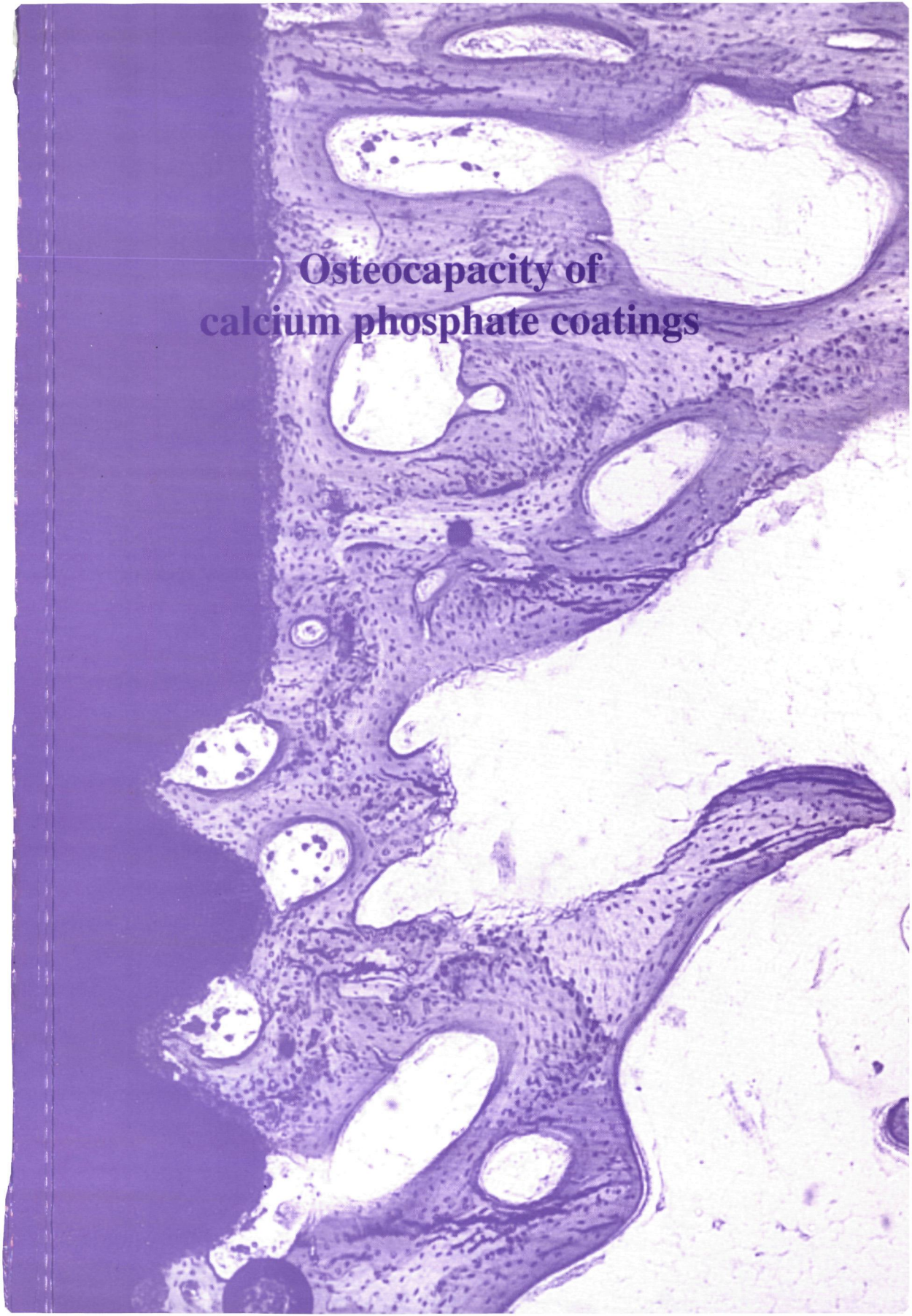
Osteocapacity of calcium phosphate coatings



Osteocapacity of calcium phosphate coatings

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* J.E.G. Hulshoff, K. van Dijk, J.P.C.M. van der Waerden, J.G.C. Wolke, W. Kalk, and J.A.Jansen, „Evaluation of plasma-spray and magnetron-sputter Ca-P-coated implants: An *in vivo* experiment using rabbits," *J. Biomed. Mater. Res.*, **31,** 329-337, (1996).

* J.E.G. Hulshoff, K. van Dijk, J.P.C.M. van der Waerden, W. Kalk, J.A. Jansen, „A histological and histomorphometrical evaluation of screw-type calcium-phosphate (Ca-P) coated implants; an *in vivo* experiment in maxillary cancellous bone of goats" *J. Mater. Sci. Mater. Med.*, **7,** 603-609, (1996).

* J.E.G. Hulshoff, T. Hayakawa, K. van Dijk, A.F.M. Leijdekkers-Govers, J.P.C.M. van der Waerden, J.A. Jansen, „A mechanical and histological evaluation of Ca-P plasma-spray and magnetron sputter-coated implants in trabecular bone of the goat," *J. Biomed. Mater. Res.*, in press, (1997).

* J.E.G. Hulshoff, J.A. Jansen, „Initial interfacial healing events around calciumphosphate (Ca-P) coated oral implants," *Clin. Oral Impl. Res.*, submitted, 1996.

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CHAPTER 1

General Introduction

1.1 Introduction

A permucosal or oral implant is an object made of a synthetic material, which is inserted nearby the mucoperiosteal tissues or in the alveolar bone, where it is intended to remain for a significant period of time to serve as an abutment for a prosthetic construction like a bridge, crown, or full prosthesis (Figures 1 and 2). The long term clinical success of oral implants is based on the presence and maintenance of a proper bone response (Zoldos, 1995). Two types of bone response to implant materials can be distinguished. The first type involves the formation of a collagenous connective tissue capsule around the implant (Weiss, 1986). Direct bone-implant contact without intervening connective tissue layer or even direct bone bonding is characteristic for the second type of bone response (Linder, 1989; Brånemark, 1985; Cook, 1992). Currently, the second type of bony fixation is considered to be the preferable situation.

One of the crucial factors in the bone bonding process is the choice of the implant material. In the last decennia numerous materials have been used for the construction of implants. Of these materials commercially pure titanium and ceramics have been proven to be most suitable.

The use of titanium for the fabrication of implants is based on the combination of the mechanical properties, corrosion resistance and biological properties (Williams, 1981; Steinemann, 1994).

Ceramics are classified as materials containing metal and non-metallic elements. For implant purposes, ceramics can be inert and interactive. Inert ceramics do not interact with the surrounding tissues and are non-soluble. An example of an inert ceramic for oral implants is aluminium oxide (Heimke, 1990). Interactive ceramics are open to intimate bonding with surrounding bony tissues. Examples are calcium-phosphates (Ca-P), like hydroxyapatite (HA). Unfortunately, a drawback is their brittleness.

1.2 History of Calcium Phosphate (Ca-P) ceramics in dental implantology

Interest in Ca-P ceramics for surgical implants is derived from their relative similarity to the mineral phase of hard tissues, such as bone, dentine and enamel. A variety of calcium phosphate salts have been distinguished, like hydroxy apatite $Ca_{10}(PO_4)_6(OH)_2$ (HA), octacalciumphosphate $Ca_4H(PO_4)_32.5H_2O$ (OCP) and tricalciumphosphate $Ca_3(PO_4)_2$ (TCP). Synthetic calcium phosphate ceramics are usually prepared by precipitation from a solution reaction at physiological pH and temperature, and subsequent sintering at 950-1200 ^oC (De Groot, 1981; Aoki, 1991).

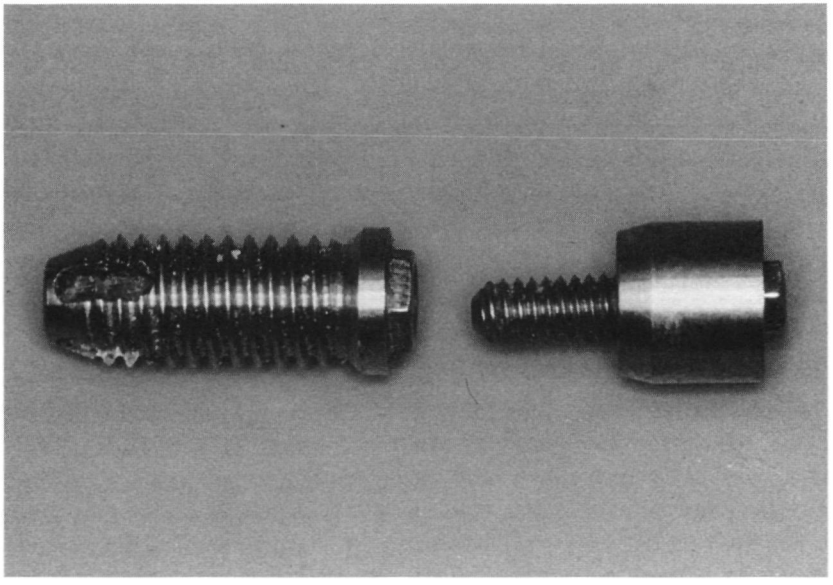

Figure 1 Photograph of a screw type implant with its belonging abutment

According to the current knowledge, the most valuable characteristic of Ca-P ceramics is their ability to become coated with a microscopic layer of bone mineral after insertion into bone tissue (Jarcho 1977, 1981). In addition, normal bone remodelling of these deposited bone layers occurs. In considering these biological advantages, however, it has to be noted that Ca-P ceramics can only conduct bone growth over the implant surface. They are not capable to induce new bone formation (Manley, 1993). Further, one of their other important aspects relates to possible transformation when they contact water. For example, hydration can change TCP to HA (Ravaglioli, 1992; Lemons, 1993).

As a logic consequence of this recognized favourable behaviour, Ca-P materials were used for the construction of dental implants. At first the use of densely sintered HA ceramics as submerged dental root implants after tooth extraction was investigated (Denissen, 1979; Cranin, 1987). In a first series of experiments, densely sintered HA blocks were inserted into artificially created bone defects in the tibiae of rats. At retrieval, after 6 months of implantation, it was found that bone tissue was growing up to the wall of the HA block. Histologically, the implanted HA block was covered with bone, even if initially a gap existed. On basis of these results, rootform HA cones were inserted into extraction sockets. The function of these implants was to maintain the width of the alveolar ridge by prevention of collapse of the buccal and lingual cortical plates. Nevertheless, despite presence of the cones, resorption of the bone continued, resulting in

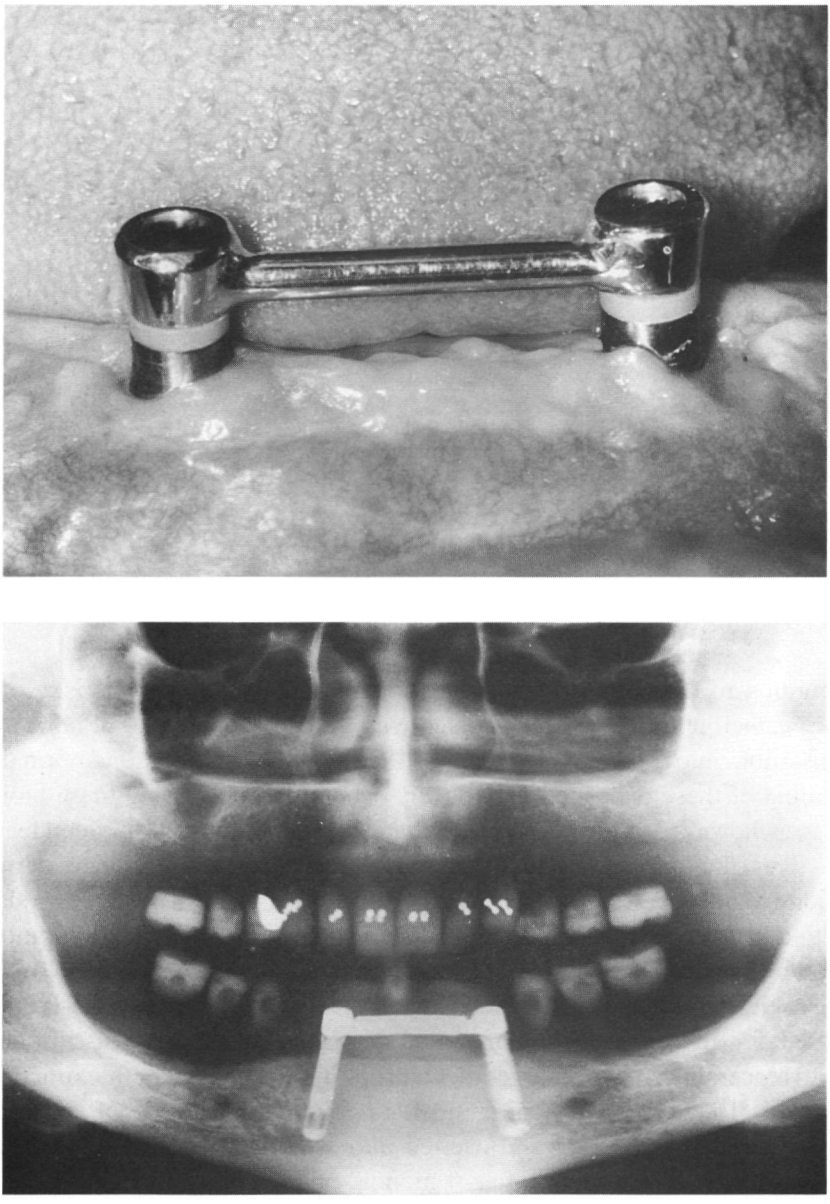

Figure 2 A Mouth of a human patient, showing a superstructure on 2 permucosal oral implants to retain a mandibular prosthesis.
B Picture of an orthopantogram, showing a mandible with 2 oral implants, superstructure and complete dental prosthesis.

exposure of the implants to the oral environment. On the other hand, it was noticed that the soft mucosal tissues showed a positive reaction to these emerged implants. Consequently, animal experiments were started to investigate the use of densely sintered HA ceramic as permucosal oral implants by de Putter (1984). Again, implants installed in the dog's jaw bones showed good fixation, adequate bone bonding and healthy gingival attachment. However, after loading the implants with crowns and bridges he found that bulk Ca-P ceramic demonstrates serious mechanical shortcomings. Although the material has high resistance for compressive forces, it shows low tensile strength. As a result fracture of all inserted implants occurred. Apparently, these bulk ceramic implants were not suited for loaded situations. Despite favourable biological properties of the implant material, these disappointing results could have been the end of the use of hydroxy apatite in loaded implant applications. Initially, de Putter tried to improve the poor fatigue properties of Ca-P ceramic by prestressing, but almost at the same time de Groot (1987) proposed to apply these materials as a coating on a metallic surface. He suggested that the excellent biocompatible properties of calcium phosphates with the good mechanical properties of metal could be combined in a better way.

1.3 Methods to deposit Ca-P coatings

To deposit Ca-P coatings on implant surfaces, various coating techniques can be used. There are:

Dip coating sintering: this process is similar to enamelling of consumer products. The metal products are dipped into a slurry containing HA powder, and consequently sintered at an appropriate time-temperature cycle to densify the ceramic coating.

Electrophoretic deposition: a process, in which charged ceramic particles suspended in solution are uniformly deposited onto the metal substrate by electrophoresis. Then the coating is densified and bonded to the metal by sintering.

Immersion coating: for this technique the ceramic is molten. Subsequently, the substrate is immersed for 3-5 sec into the melt.

Hot isostatic pressure: a method in which heat (1000-2000oC) and pressure (10.000-15.000 psi) are combined during sintering of the powder compact. The coating contains a higher density than can be obtained by conventional sintering.

Sputter-deposition: is the process whereby atoms or molecules of a material are ejected from a target by the bombardment of high-energy particles. There are several sputtering techniques, e.g. cathodic sputtering, diode sputtering, radiofrequency (RF) or direct current (DC) sputtering, ion-beam sputtering, reactive sputtering.

Plasma spraying: is a technique in which the ceramic particles are partially molten by being propelled through a high temperature plasma so that they fuse together to form a coating upon striking the surface of the substrate. Heating of the substrate material to a full sintering temperature of the ceramic powder is not required.

With these methods, coatings can be deposited with thicknesses between a few micrometers and a few millimetres.

Various studies (Dhert, 1992; Clemens, 1995; Lacefield, 1988; Koeneman, 1989) have demonstrated that all of the above mentioned methods suffer their own disadvantages and limitations:

* dipcoating sintering and electrophoretic deposition negatively change the mechanical properties of the coated metal substrate, because of the required heat at sintering of the coating. Besides that the adherence of the coating to the substrate surface is insufficient.
* immersion coating causes undesirable structural changes of the ceramic, and again an insufficient coating substrate adherence is shown.
* the HIP method uses a changeable mould to press and heat the coating. The material of the mould influences and reacts with the produced coating. Undesired changes of the composition of the coating are shown. Further, it is an expensive technique.
* disadvantages of the plasma spray technique are: (1) a non-uniform density of the coating, (2) the high plasma temperature changes the instable phases of the used ceramic powder, and (3) porosity at the interface substrate/coating can appear. An advantage of plasma spraying is that sintering after coating is not required, so the substrate material is not exposed to the produced heat of sintering.
* sputter coating, produces dense, adherent ceramic coatings. Mechanical properties of the substrate material are not influenced by this procedure. Only very thin (1-2 μm) coatings can be produced in a reasonable time of coating.

The above mentioned does not exclusively direct to the preferred technique of producing a dense, adhesive ceramic coating for an implant material. Generally, it is supposed that plasma-spraying and sputter-coating are the most suitable methods for producing HA-films on implants. At the moment, most laboratory investigations are directed to the further development of the plasma-spray procedure.

1.4 Plasma spray Ca-P coatings

Currently the plasma spray technique has been used widely for biomedical applications such as dental root implants (Wolke, 1992a; Dalton 1995). A more detailed description of the plasma-spray technique is given by Herman (1988). The plasma-spray process requires roughening of the metallic implant surface e.g. by grit blasting, in order to obtain mechanical retention of the coating. The principle of the plasma spray procedure of ceramics has been derived from standard available techniques. A schematic drawing of the plasma spray process is given in Figure 3. In a so called plasma-gun an electric arc current of high energy is struck between a cathode and an anode. An inert gas is directed through the space between these electrodes, subsequently the arc current ionizes the gas and a plasma is formed. In this plasma, the electrons and ions are separated from each other and are accelerated towards the cathode and anode respectively. These rapidly moving particles then collide with other atoms or molecules in the gas which results in expansion due to the temperature increase. Then a plasma-flame is formed which emerges from the gun towards the substrate. This plasma-flame approaches the speed of sound. Next, ceramic powder particles are fed into the plasma flame. The particles melt and are deposited on the substrate at which the gun is aimed. The quality of plasma spray deposited coatings can be influenced by several parameters such as temperature of the plasma, nature of the plasma-gas, particle size of the powder and chemical nature of the ceramic powder (Wolke, 1992b; Clemens, 1996). In this context, it has to be recognized that the Ca-P coatings deposited by the plasma process are quite different to bone mineral apatite. During plasma-spraying, overheating and melting can change the synthetic HA powder. As a result, the deposited coating will consist of HA, as well as amorphous and other crystalline Ca-P components (Zyman, 1993, 1994; Tong, 1996). Therefore, it has been suggested that all manufacturers of HA-coated implants have to perform relevant chemical and analytical tests to assure the quality of their coatings (Kay, 1992; Dalton, 1995). This analysis should include information about: chemical composition, Ca/P ratio, crystallinity, density, tensile strength, thickness and uniformity, and trace element analysis.

Nevertheless, irrespective of the apparent importance of such an accompanying report, these control tests will be no guarantee for the final biological performance of the coated implants. For example, although a higher crystallinity will decrease the extent of coating dissolution, faster bone bonding is probably supported by coatings with high levels of more soluble amorphous phases (Jarcho, 1992; Jansen, 1993a; van Blitterswijk, 1993; De Bruijn, 1993). Therefore, also biological evaluations have to be made of such certified coatings.

Currently all the above mentioned information is not available. Consequently, comparison between plasma-spray Ca-P coatings produced by different

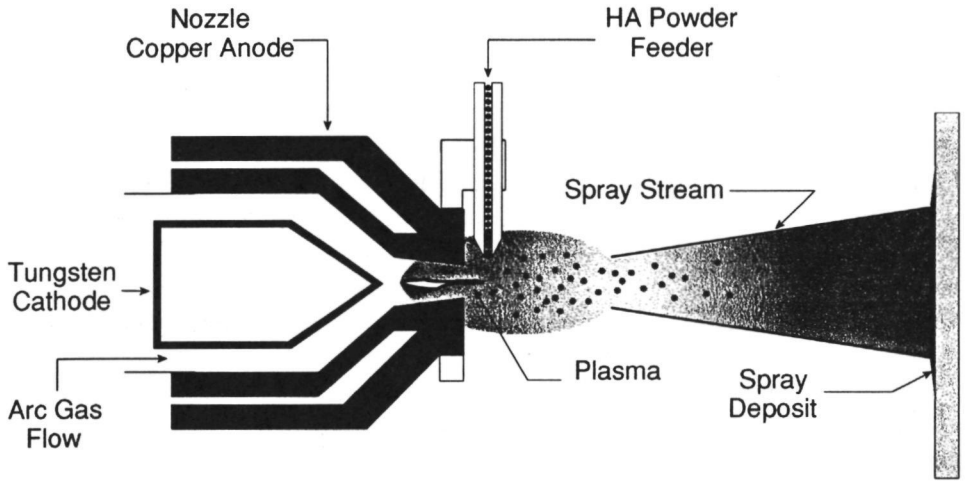

Figure 3 Schematic drawing of the plasma spray process

manufacturers is very difficult. Still various experimental animal studies, as performed up to now, demonstrated a faster and greater bone adaption to plasma-spray Ca-P coated implants (Kay, 1992; Weinlander, 1992; Jansen, 1993a; Caulier, 1996; Klein, 1991; Klein, 1994; Geesink, 1987; Søballe, 1993; Dhert, 1993; Gottlander, 1992; Cook, 1988; Kitsugi, 1996; Evans, 1996). The obtained histological results show: (1) higher percentages of bone contact along Ca-P coated implants compared to non-coated implants, and (2) greater stability, as measured by higher fixation strengths after short and prolonged implantation periods.

1.5 Clinical perspective of Ca-P coated oral implants

The promising results from the earlier mentioned animal studies formed the base for the use of Ca-P coated implants in human patients. However 10 years after their introduction, still most studies only report about the first 5-6 years of clinical performance of such HA-coated implants (Block, 1992; Kent, 1990; Golec, 1992; Stultz, 1993; Saadoun, 1993; Babbush, 1993; Krauser, 1989; O'Roark, 1991; Hahn, 1990; Kirsch, 1989; Ochi, 1994; Wheeler, 1996; Fugazzotto, 1993; Jemt, 1996).

By reviewing these studies a problem arises. Unfortunately, there is no generally accepted standard for success of an oral implant. Failure of an implant can be identified by clinical and radiographic symptoms, but the criteria for success are less evident. As a result, in some studies the criteria are used as proposed by Albrektsson (Albrektsson, 1986), i.e.:

1. immobility of an individual, unattached implant.
2. no radiographical evidence of peri-implant radio lucency.

3. less than 0.2 mm annual vertical bone loss following the implant's first year of service.
4. absence of persistent and irreversible symptoms as pain, infections and paraesthesia.

In contrast, other studies considered the survival of the implant as being the most important evaluation criterium. Survival was defined as an implant functioning successfully as a support for a prosthesis. Failure was defined as an implant that failed to integrate, or any implant that failed during functioning and had to be removed. Consequently, there is an urgent need to define the diagnostic factors for evaluation of endosseous oral implants, in order to determine the predictive value of some indices in longitudinal studies. Keeping these considerations in mind, a review will be given.

Following the initial animal studies, in 1984, the first HA coated dental implants were sold under the name Integral®. These titanium alloy implants were provided with a 75 µm thick layer of hydroxy apatite. Since then, several clinical trials have been performed, with apparently satisfactory results.

For example, Block (1992) and Kent (1990) reported about the 5-year evaluation of 740 HA-coated implants placed in 215 patients. Implants were inserted both in the maxilla and mandible. The cumulative 5-year success rate for all implants was 91.74%. In a separate study, they placed 62 HA-coated implants in fresh extraction sites immediately following the removal of the tooth. After follow-up of 3-5 years, these implants showed the same success rate as those placed in edentulous bone.

In another study, Golec (1992) reported about a retrospective evaluation of 1085 patients treated with 3093 HA-coated Integral® implants. The implants were positioned in the maxilla, mandible and immediate extraction sockets. The 5-year survival rate for all implants was 97%. Loosening of the implants because of failure of the coating-metal interface was not observed in any patient within this study.

However, the most extensive study with HA-coated Integral® implants is performed by Stulz (1993). In this multicenter analysis, twelve centres participated. In these centres 6203 implants were placed in 2104 patients. In total 3693 implants were placed in the mandible and 2371 implants in the maxilla. The location of 139 implants was unknown. The cumulative survival rate at 5 years for mandibular implants was 95.7% and for maxillary implants 93.2%. Most failures occurred of implants in the posterior regions, 28.9% for the posterior maxilla and 35.9% for the posterior mandible. This high rate of failures for the posterior implant positions was attributed to: poor bone quality, proximity of vital anatomic structures and higher occlusal forces than compared with those in the anterior area. Most failures (51 of 146) were early implant failures due to failures in the initial integration or during the first year of service.

In addition to the Integral® system, other HA-coated implants were introduced like Steri-Oss® and IMZ®.

Saadoun (1993) described the results of 673 Steri-Oss® implants placed in 280 patients. Of these, 241 were titanium screws, 104 were titanium screws coated with hydroxy apatite and 328 were titanium cylinders coated with hydroxy apatite. The implants were located in the maxilla and mandible. A comparison of the 5-years results of the different types of implants revealed an increasing success rate from a low of 85.05% with the pure titanium screws, to 97.14% with the HA-coated titanium screws to as high as 98.48% with the HA-coated titanium cylinders. Comparison of the success and failure rates of maxilla versus mandible revealed that the mean success rate of 94.5% for the mandible drops to 90.3% for the maxilla. Further, the success rates are higher in the anterior than the posterior areas of the jaw arches.

Babbush (1993) reported about patients who were reconstructed with coated and non-coated IMZ implants. Of the 1059 implants inserted during a 5-year period, 124 (12%) were provided with an HA-coating. Of these coated implants, 59 implants were used in the maxilla and 65 in the mandible. During the evaluation period, only four of these failed. Therefore, the success rate is 96.8%, which was comparable with the overall survival rate of the non-coated implants.

Finally, Wheeler (1996) presented an 8 year evaluation of 5 different types of titanium plasma-sprayed and hydroxy apatite coated cylinder implants. A total of 1202 implants were placed. Of these, 313 implants were provided with an HA coating; 134 in the maxilla and 179 in the mandible. After 8 years the overall survival rates for the maxilla and the mandible were 80.26% and 90.75% respectively. Life table analysis showed significantly higher success rates of HA coated implants in the first 2 years. However after 8 years, cumulative survival rates for titanium plasma spray coated systems compared to HA coated systems seemed better (92.8% versus 79.2% in the maxilla). Still, it has to be emphasized that this observed difference was not statistically significant.

For general acceptance and clinical use of Ca-P coated implants, it is important to note, that reported data have to be confirmed by analysis of HA-coated implants, that have been retrieved from human patients. Fortunately, already a few histological studies of HA-coated implants under loaded conditions are available (Piatelli, 1993a,b; Oguchi, 1994). Examination of the bone-implant interface of these retrieved implants showed very intimate bone HA-coating contact. Further, it was found that the bonding of the HA-coating to the metal was strong enough to resist loading forces.

When compared with the clinical data from studies with non-coated implants the results of the above mentioned studies appear to confirm the efficacy of HA-coated implants at sites with an insufficient quantity and or quality of the bone. On

the other hand, presented data also seem to justify some concern. Therefore, in order to get more insight into the performance of Ca-P coated implants in low quality maxillary bone, Caulier (1995, 1996) performed animal experiments to study the real contribution of Ca-P coatings to the bone response. Two questions were investigated: (1) Does the application of Ca-P coatings improve the success rates of permucosal implants in a low quality type of bone? (2) Does the application of Ca-P coatings reduce the required intervening healing time before loading of enossal oral implants?

In this study Ca-P coated and non-coated Brånemark® dental implants were inserted in the maxilla of goats. After a healing period of 6 months, the implants were exposed and provided with permucosal abutments. The animals were killed 4 months after installation of the abutments. The results with these coated and non-coated Brånemark® implants were surprising. During placement of the abutments, it appeared that of the 16 placed non-coated implants, 6 were lost or too mobile for abutment installation. During the permucosal phase an additional loss of 3 implants occurred. Of the 48 Ca-P coated implants only 4 were lost during the enossal stage and 3 during the permucosal stage. Therefore, the success rate of the non-coated implants was 43.7% and 87.5% for the Ca-P coated implants. Besides, Periotest® measurements demonstrated that the fixation of all maintained non-coated implants was decreased during the 6 months healing period, while all maintained Ca-P implants showed an increased bone fixation. In an additional experiment, 48 implants were positioned in the maxilla of 12 goats. Healing of plasma spray coated and uncoated implants was evaluated after 3 and 6 months of implantation. On basis of the results Caulier suggested that it is probably not necessary to wait longer than 3 months before loading maxillary implants, when the implants are provided with an amorphous plasma-spray HA coating. This hypothesis corroborates with the observations of Lum (1992). In a human patient a torquing device was used to measure the shear resistance of the supporting bone. It was found that the bone was capable of resisting shear forces just slightly greater than that of which is necessary to displace implants inserted in dog femurs with comparable bone-healing period and with healing to full maturity. Therefore, he also suggested that routine early uncovering of two-stage HA-coated implants is feasible, in this case report even after 2 months of implantation.

1.6 Clinical Concerns about plasma spray Ca-P coatings

Despite all encouraging results, still concern and confusion exists, regarding the viable use and prognosis of plasma spray HA-coatings (Kangasniemi, 1994; Clemens, 1995; Wolke 1996; Hulshoff, 1996; Caulier, 1996; Kitsugi, 1996). These concerns deal with:

1. the substrate-to-coating fracture and fatigue strength properties.
2. the biodegradation of the coating and what will happen at the implant-bone interface when the coating has disappeared.
3. the length of the HA-coating in relation to the required surface texture of the top of the implant.

For example, as described in section 1.5, besides increasing, also decreasing success data with HA-coated implants are reported (Wheeler, 1996). This is attributed to extensive cervical bone loss. On the other hand, it has to be emphasized that severe bone loss can also be caused by functional overload, differences in the various types and configurations of implants, as well as the fit and design of the superstructure (Meffert, 1992). Consequently, for a definite answer on this controversial topic systemic and controlled studies, examining all the various parameters which can influence the bone implant reaction, have to be performed.

However, besides this research to optimize the clinical application of Ca-P coated implants, another goal has to be the further improvement of the coating characteristics. In view of this, in our laboratory experiments (Jansen, 1993b) were started to the efficacy of RF magnetron sputtering for the deposition of Ca-P coatings.

1.7 About the RF magnetron sputter technique to produce Ca-P coatings

As mentioned in section 1.3, sputtering is the process whereby atoms or molecules of a material are ejected from a target by the bombardment of high-energy particles. There are several sputtering techniques, e.g. cathodic sputtering, diode sputtering, radiofrequency (RF) or direct current (DC) sputtering, ion-beam sputtering, reactive sputtering. All these techniques are variants of the above mentioned physical phenomenon. The process of sputtering is very slow; which is considered as a serious drawback. By using the magnetically enhanced variant of diode sputtering, the so called RF magnetron sputtering method, the process can be accelerated. A schematic drawing of the vacuum chamber at which the sputter process takes place is given in Figure 4. Argon gas is conducted into the vaccuum chamber at which the sputter process takes place. Ar^+-ions strike the target, that serves as a source material, and eject neutral target atoms through momentum transfer. These atoms enter and pass through the discharge region to eventually deposit on the substrate material, in this way forming the growing coating. In addition, other particles (secondary electrons, desorbed gases and negative ions) are emitted from the target. On their way through the discharge the electrons emitted from the target will ionize argon atoms, which will increase the

productivity of the argon discharge. The magnets, positioned under the target material create a magnetic field above and parallel to the target surface in which the electrons are trapped. This increases the probability that these electrons will collide with and ionize a gas molecule, and therefore accelerate the sputtering process.

Magnetron sputtering has to be considered as a high-rate vacuum coating technique for depositing a wide range of materials (including metals, alloys and ceramics). The primary advantages of magnetron sputtering are:

1. excellent adhesion of coating
2. thickness uniformity
3. the ability to coat implants with difficult surface geometries
4. ease of automation

By using sputter-deposition techniques, more dense and adherent coatings can be obtained, than with e.g. the plasma spray technique.

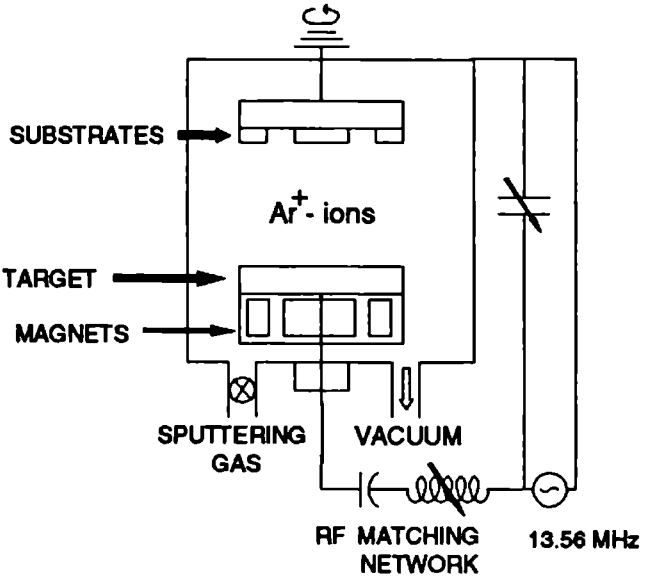

Figure 4 Schematic drawing of the magnetron sputter process

1.8 Objective of the study

In a preliminary study (Jansen 1993b) RF magnetron sputtering was used to produce Ca-P coatings. Scanning electron microscopy revealed a dense columnar structure and uniform thickness of the deposited coatings. Various physico-chemical analysis methods demonstrated well crystallized Ca-P ceramic with a Ca/P ratio varying between 1.9 and 2.5, and pilot biological experiments confirmed biocompatibility of the RF magnetron sputter coatings. Still several issues have to be solved before the RF magnetron sputter method can be used to produce pure crystalline Ca-P ceramic coatings on a routine basis. Therefore, further *in vitro* and *in vivo* studies have to be performed.

Above mentioned formed the base to start the experiments as described in this thesis. The overall objective of this study was to evaluate the biological characteristics and suitability of RF magnetron sputter Ca-P coatings for oral implants.

Approach:

Considering the aim of this study the following experimental questions were addressed:

* Do RF magnetron sputtered Ca-P coatings induce a similar or better bone behaviour than plasma-spray Ca-P coatings? To answer this question at first „simple" *in vitro* and *in vivo* experiments with osteoblast like cells and in the trabecular bone of the rabbit were performed. The results supported us to proceed with experiments into more difficult conditions, i.e. the maxillary trabecular bone of the goat.
* Do RF magnetron sputter Ca-P coated implants perform different compared to plasma spray coated implants at loading till failure? A well controlled method to apply and measure a torsional force to load screw type implants to the point of failure was introduced.
* Do RF magnetron sputter Ca-P coatings and plasma spray Ca-P coatings influence the initial healing of implants inserted into trabecular bone? Differently coated implants were evaluated after 3, 12 and 24 days of implantation in trabecular bone of the femur of the goat.
* Do RF magnetron sputter coated substrates influence formation of extra cellular matrix by osteoblast like cells? Substrates with different Ca-P ratio were examined *in vitro*. Besides quantitative analysis, morphology at the ultrastructural level was evaluated.

1.9 References

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CHAPTER 2

Biological evaluation of the effect of magnetron sputtered Ca-P coatings on osteoblast-like cells *in vitro*

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2.1 INTRODUCTION

Since about 10 years, the application of calcium phosphate (Ca-P) coatings has become an accepted technique to improve the biologic behavior of metallic dental and orthopaedic implants (Geesink, 1993). Currently, plasma-spraying is the most frequently used method for the deposition of Ca-P coatings on implant materials. However, despite the described beneficial effect of these coatings on the bone response (Caulier, 1996; Klein 1994; Søballe 1993; Dhert 1993; Gottlander, 1992), there is also some concern regarding their long term performance (Kay, 1992). For example, it is reported that the Ca-P coating resorbs over time and loses its mechanical integrity by lack of adherence (Kangasniemi, 1994). Since the clinical consequences of these findings still have not been understood completely, more knowledge and experience has to be obtained about the characteristics of Ca-P coatings. To achieve this goal, besides the further improvement of the plasma-spray technique, the efficacy of more appropriate deposition methods has to be investigated. Therefore, in our laboratory experiments have begun on the application of magnetron sputtering for the production of Ca-P coatings on metal and plastic substrates.

Radiofrequency (RF) magnetron sputter-coating is performed using commercially available sputter equipment. In a preliminary study, we showed that this technique produces highly adhesive, uniform coatings (Jansen, 1993). Energy-dispersive X-ray analysis, X-ray diffraction and atomic absorption spectrometry confirmed that the sputtered layers were well-crystallized Ca-P ceramic with a Ca/P ratio varying between 1.9 and 2.5. *In vitro* and in *vivo* experiments demonstrated the biocompatibility of the coatings. Based on these results, we concluded that magnetron sputtering is a promising method for forming a Ca-P coating onto an implant material. Nevertheless, several problems, e.g. the endurance and the Ca/P ratio of the coating, have to be solved before clinical use of magnetron sputtered implant systems can be considered. To elucidate these problems and to gain more insight into the physico-chemical and biological properties of the sputtered coatings extensive *in vitro* and in *vivo* experiments have to be done. In this article we present the first stage of the biological evaluation by using rat bone marrow cell cultures to investigate the potential of Ca-P sputtered coatings for osteoblast-like cell growth.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Commercially pure titanium (cpTi) discs with a diameter of 12 mm were used.

They were polished to 320 grit with abrasive papers, ultrasonically cleaned for 15 min. in acetone and placed in 100% boiling ethyl alcohol. After drying, the discs were left untreated or provided with four different Ca-P coatings. Two types of Ca-P coatings were deposited using a plasma-spray technique. The first was used as applied (HA-PS), the second was subjected to a heat treatment for 2 hours at 600 °C after coating deposition (HA-PS/ht). The thickness of these coatings was about 50 µm.

Two other types of Ca-P coating were produced using the RF magnetron sputter technique. The sputter process was done at a power level of 800 W and a process pressure of $5x10^{-3}$ mbar using argon gas. The titanium discs were mounted on a water-cooled substrate holder. One coating was produced with a rotating substrate holder (Ca-P-a), while the other coating was deposited with the substrate holder in an indexed position (Ca-P-c). The thickness of the magnetron sputtered coatings varied between 2.5-4.0 µm.

All deposited coatings were characterized by scanning electron microscopy (SEM), X-ray diffraction (XRD) and Fourier infrared absorption spectrometry (FTIR).

Before use in the cell culture experiments, all discs were autoclaved for 30 min. at 120 °C.

2.2.2 Cell isolation and culture

Osteoblast-like cells were prepared using the rat bone marrow (RBM) culture method as described by Maniatopoulos (1988), Davies (1990) and De Bruijn (1992).

Briefly both femora of young adult male Wistar rats (weight 100-120 g, age 40-43 days) were removed and washed four times with α-Minimal Essential Medium (MEM, Gibco, Life Technologies B.V., Breda, The Netherlands), containing 0.5 mg/mL gentamycin (Gibco) and 3.0 µg/mL fungizone (Gibco). Afterwards, the epiphyses were cut off and the diaphyses flushed out, using α-MEM supplemented with 15% fetal calf serum (FCS, heat induced at 56 °C for 35 minutes, Gibco), 50 µg/mL of freshly prepared ascorbic acid (Sigma, Chemical Co., St. Louis, MO., USA), 10 mM Na ß-glycerophosphate (Sigma), 10^{-8}M dexamethasone (Sigma) and antibiotics at 1/10th of the concentration described above. Finally, cultures were incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C. The phase contrast photomicrograph of Figure 1 shows the general appearance of a primary culture. After 5 to 7 days in culture, cells were harvested by trypsin treatment (0.25% w/v trypsin) and used for the experiments.

The osteogenic phenotype and function of the RBM cells were confirmed by several parameters, including the presence of alkaline phosphatase, deposition of calciumphosphate material by Von Kossa's method and immunostaining. For the

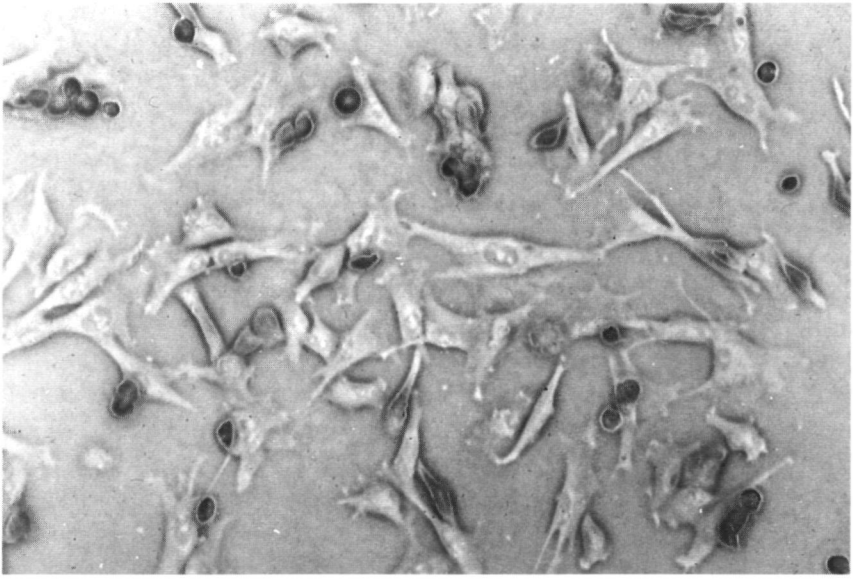


Figure 1 Phase contrast micrograph of a primary rat bone marrow cell culture.

immunocytochemical identification we used a specific monoclonal antibody E11 (Wetterwald, 1996) against a cell membrane associated antigen of rat osteoblasts. A goat-antimouse/FITC conjugate was used to locate the monoclonal antibody. We noted that 12 day-old cultures on microscopic glass slides showed a positive anti-osteoblast reaction. No attempts were made to detect the possible influence of different substrates on the osteoblast expression of the cell cultures.

2.2.3 Cell attachment assay

The test substrates were positioned on the bottom of sterile 24-well plates (Greiner, Greiner B.V., Alphen a/d Rijn, The Netherlands) and a total of 1.0 mL of culture medium containing $5x10^4$ cells was added to each substrate. The cultures were incubated for 8 hours at 37 °C in 5% CO_2/air. After incubation, the wells were washed twice using phosphate buffered saline (PBS) to remove non-attached cells. Then, the substrates were taken out of the wells and placed into counting tubes. To detach the attached cells, 1 mL of trypsin was added and the tube was placed for 8 minutes at 37 °C. Isotone solution was added and the cells were counted using a Coulter Counter. After the counting procedure the presence of nondetached cells was checked by scanning electron microscopy of the various substrate surfaces.

Two runs of experiments were carried out. In each run all materials were present in quintuple.

2.2.4 Cell proliferation assay

RBM cell suspensions, containing $5x10^4$ cells, were seeded on the experimental substrates as described before and incubated at 37 °C in 5% CO_2/air. After 8 hours of incubation, the nonattached cells were removed by PBS rinses. To each well 1 mL of fully supplemented medium was added and the specimens were incubated for 5 days. The medium was changed every other day. At the end of the incubation period, the medium was discarded and the substrates were rinsed twice with PBS. Then, the substrates were taken out of their wells and placed into counting tubes. After detachment by trypsinization, isotone solution was added to count the number of adherent cells using a Coulter Counter. Similar to the attachment assay, non-coated and coated surfaces were checked on the presence of non-detached cells.

The presented results are based on the average of two separate experiments. In each experiment all materials were present in quintuple.

2.2.5 Cell morphology assay

RBM cell suspension (200 µL per well, containing $1x10^4$ cells) was added to the test substrates as previously described for the cell attachment and proliferation experiments. The cultures were incubated for 6 and 18 days at 37 °C in 5% CO_2/air. The culture medium was changed every other day. After the various incubation periods, the non-attached cells were removed by PBS rinses. The attached cells were fixed in situ with 2% v/v glutaraldehyde in 0.1 M sodium cacodylate buffered solution for 30 minutes at 4 °C, rinsed twice in cacodylate buffered solution, followed by dehydration through a graded series of ethanol. Subsequently, the specimens were dried by tetramethylsilane. Finally, after sputter-coating with gold, they were examined using a Philips SEM-500 scanning electron microscope at an accelerating voltage of 12 kV.

The possible influence of culture medium on the non-coated and Ca-P coated titanium discs was also examined. Therefore, some discs were incubated for 6 and 18 days with fully supplemented culture medium, but without cells. After incubation, the discs were processed for scanning electron microscopy.

2.3 RESULTS

2.3.1 Characterization of the Ca-P coatings

A complete description of the characteristics of the coatings has already been given elsewhere (Wolke 1992; Wolke 1994).

In summary, SEM examination revealed that the specimens were covered with a uniform coating. Further, it was observed that the Ca-P-a coatings had a smoother surface microstructure, while the Ca-P-c surfaces showed a polycrystalline appearance.

XRD patterns demonstrated that the HA-PS and HA-PS/ht coatings showed an amorphous/crystalline structure. The crystallinity of the HA-PS coatings appeared to be 60 %. After heat-treatment (HA-PS-ht specimen) the crystallinity of the coatings increased slightly to 65 %.

In Figure 2 XRD patterns of the Ca-P-a and Ca-P-c are shown. The analysis of the diffractograms showed that the indexed procedure resulted in a crystalline Ca-P coating with a preferred (00l) crystallographic orientation with the C-axis perpendicular on the substrate surface (reflections 002, 102, 112, 202, respectively 25.9, 28.1, 32.4 and 34.0° 2-Theta). Coatings prepared with the rotating substrate holder showed an amorphous structure, without any specific reflection lines.

Infrared measurements of the plasma-sprayed samples showed absorption bands characteristic of P-O and O-H bonds. In the spectra of both sputtered coatings no OH bonds were detectable. These coatings showed a wide peak over the region from 2800 to 4000 cm^{-1} indicative for water absorption at the surface (Elliot, 1973).

2.3.2 Cell attachment

Table 1 shows the results of the osteoblast attachment experiments. Statistical testing of the findings, using an one-way analysis of variance (ANOVA) and a multiple comparison procedure (Newman-Keuls) revealed that no significant differences existed between the attachment percentages of RBM cells to the various materials. Data are unavailable for Ca-P-c coatings due to detachment of the coating during cell trypsinization, which obstructed the orifice of the counting tube. SEM inspection demonstrated that no cells were left on the various substrates after trypsinization.

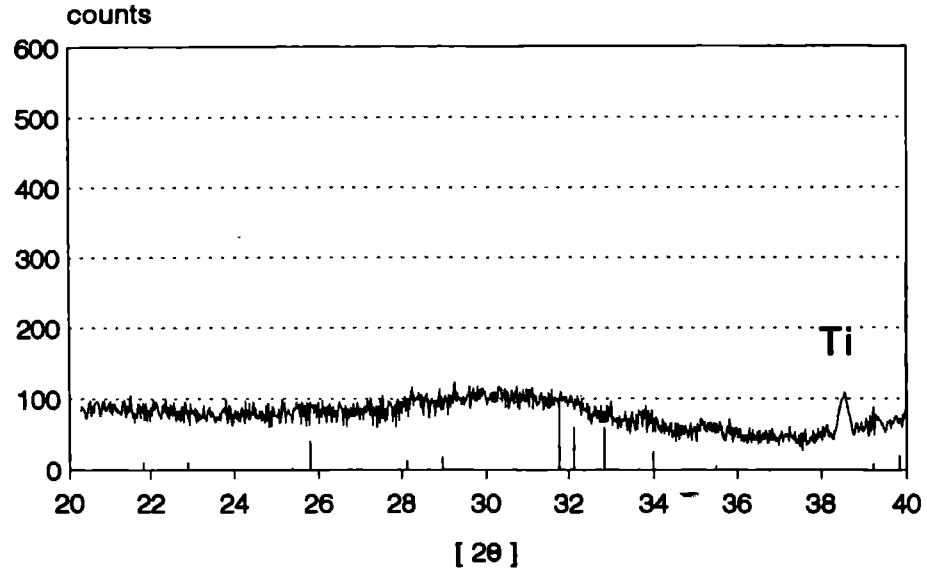
2.3.3 Cell proliferation

The results of the proliferation assay of RBM cells on the various substrate materials are given in Table 1. SEM examination of the trypsinized substrates, after the 5-day culturing period of the proliferation assay, demonstrated incomplete removal of the cells from HA-PS-ht surfaces (Figure 3). Therefore, these data were excluded from the statistical analysis. One way analysis of variance (ANOVA) and multiple comparison procedure (Newman-Keuls) revealed no significant difference between titanium and the other materials.

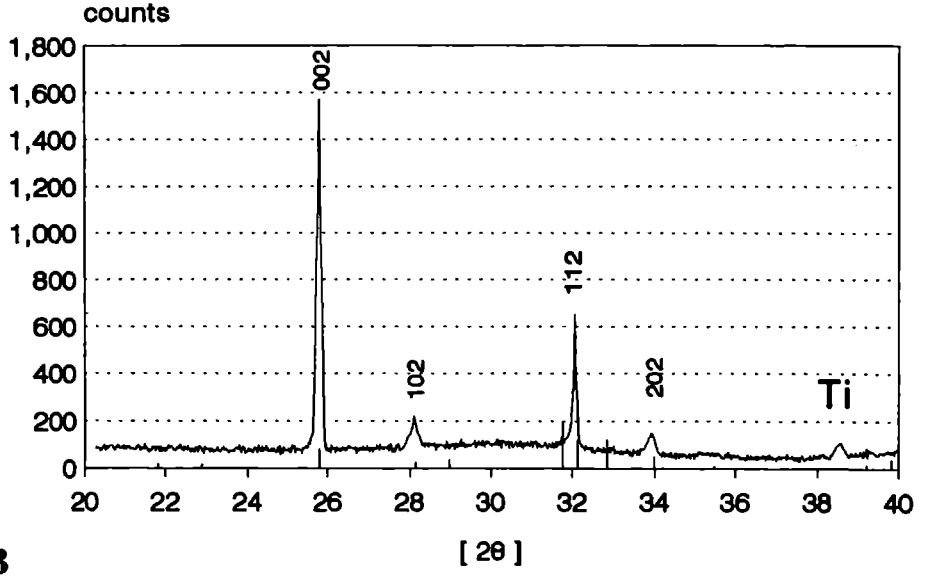
2.3.4 Cell morphology

Scanning electron microscopy showed a comparable cell morphology on all test surfaces. Cells cultured on cpTi surfaces formed multilayers. No systematic signs of bone-like tissue formation were observed (Figure 4).

The morphology of RBM cells cultured on the different plasma-sprayed coatings was similar as described by De Bruijn (1992). In short: after culture



A



B

Figure 2 X-ray diffraction pattern of the magnetron sputtered coatings;
A. amorphous Ca-P-a coating.
B. crystalline Ca-P-c coating.

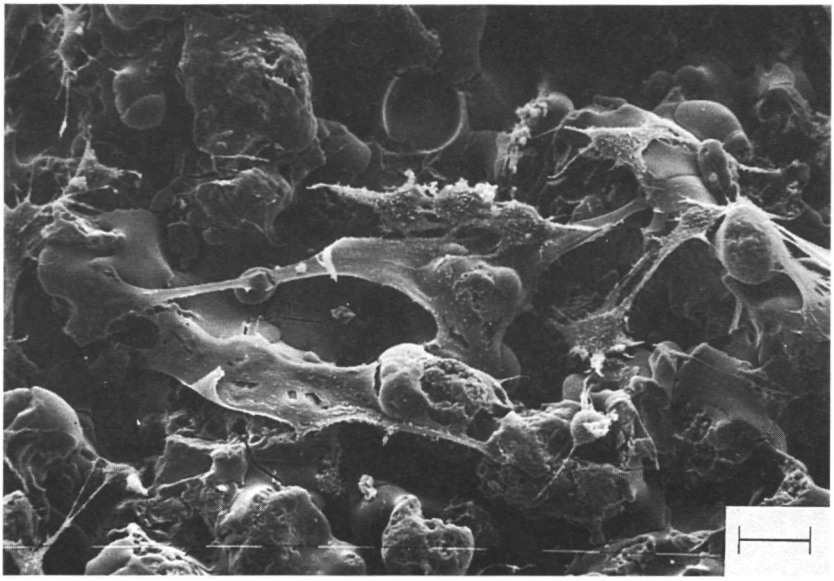


Figure 3 Scanning micrograph showing cells left on a plasma-spray coated substrate left after trypsinization, bar = 12.8 µm.

periods of 6 days the cells were spread and exhibited filopodia spanning the macropores produced by the plasma-spraying. Extracellular matrix formation was observed after 18 days of culture. No difference in coverage with extracellular matrix existed between the heat treated and non-heated plasma-sprayed coatings.

The cellular response to the sputter-coated ceramic substrates was identical to the plasma-sprayed substrates. Although, the occurring processes were easier to

TABLE 1
Numbers of attached and proliferated rat bone marrow cells cultured on the various substrate materials

RESULTS:	attachment		proliferation	
substrate	mean	SEM	mean	SEM
cpTi	22624	272	218322	218322
HA-PS	22400	1408	226395	38545
HA-PS/ht	23928	1000	145743	13117
Ca-P-a	28736	2736	255854	53650
Ca-P-c	–	–	249080	53846

SEM represents standard error of the mean

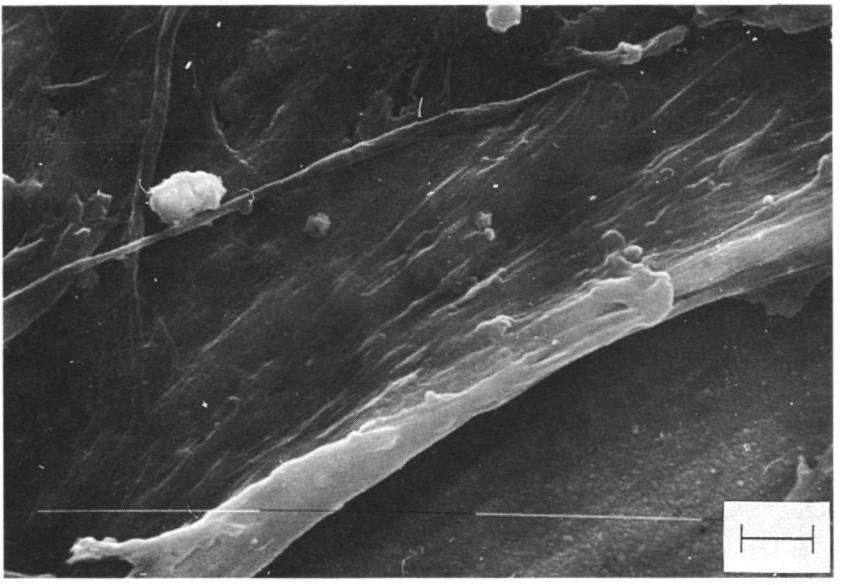


Figure 4 Scanning electron micrograph showing a multilayer of RBM cells including the underlying titanium surface. Although some globular interfacial matrix formation can be observed, it has to be noted that this matrix was not systematically present, bar = 3.2 µm.

follow due to the smooth surface texture of the sputtered coatings. Following incubation, the RBM cells adhered and spread over the sputtered Ca-P surface, resulting in a confluent cell layer at the 6th day. After 18 days formation of a granular mineralized layer was seen. No differences in appearance and coverage of this ECM layer was observed between both types of sputtered Ca-P ceramic (Figure 5 and 6). Examination of cracks in this layer, which were due to the drying process, made apparent that this layer was rather thick (about 1 µm). In areas where parts of the ECM were elevated, afibrillar mineralization foci were observed. In addition, exposure of the underlying ceramic surface proved that 18 days after incubation a substantial thickness of the coating was still present. Further inspection, revealed the precipitation of tiny spherulites (about 0.35 µm) covering this original coating surface.

This formation of spherulites on the surface of the sputtered coatings was also seen after 6 days of incubation in fully supplemented culture medium without cells. Figure 7A and 7B shows the SEM photograph of these specimens. The small cracks, present in the coatings are result of the SEM preparation process. This kind of spherulites was also formed on the control HA-PS specimens, while on the heat treated plasma-sprayed surfaces deposits did not develop. After 18 days of incubation, these spherulites increased in number, as well as in size, on the Ca-P-a,

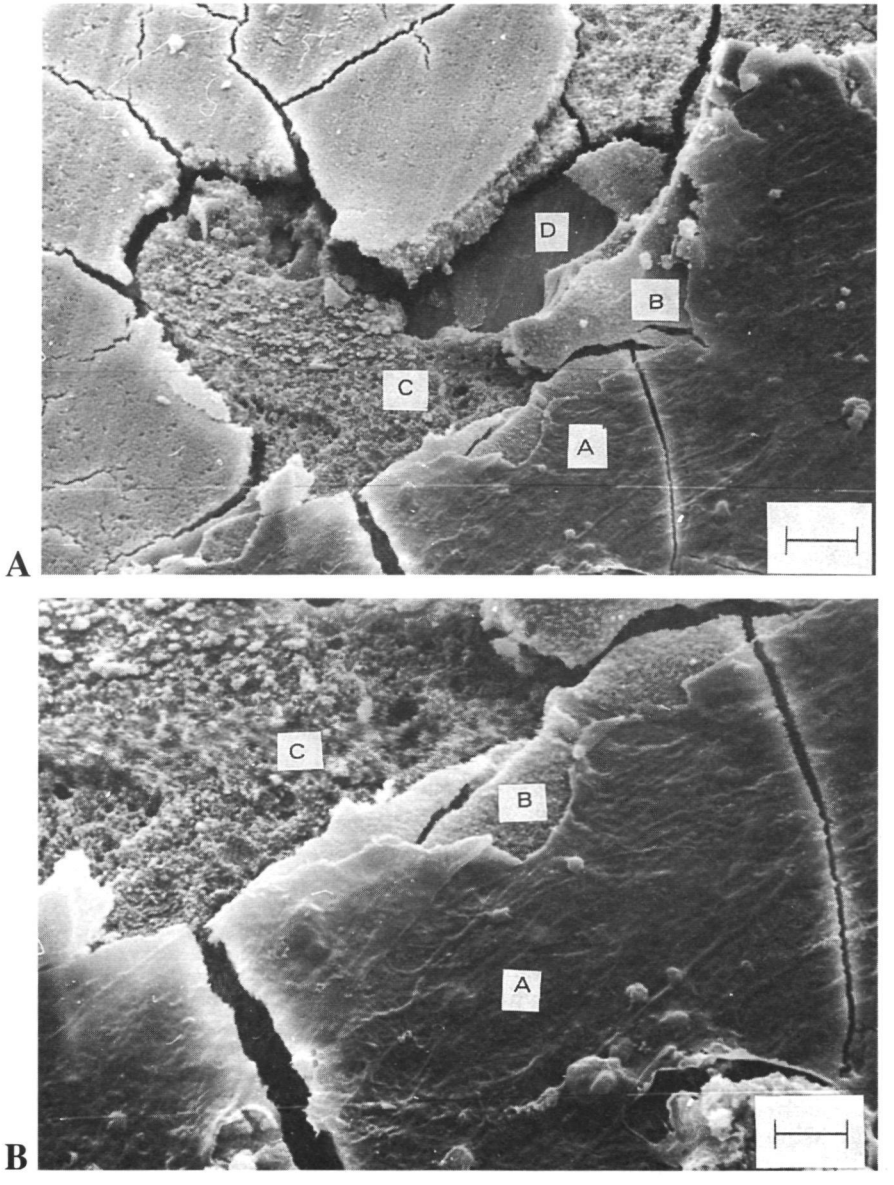


Figure 5 Scanning micrographs of RBM cells cultured for 18 days on a Ca-P-a coated specimen. Four different layers can be observed; A: RBM cell multilayer, B: granular mineralized layer, C: Ca-P-a coating, and D: original titanium surface. The coating is covered with small spherulites. The specimens show numerous cracks. As shown by the continuation of cell structures at both sides of these cracks, they are caused by the drying method use for SEM preparation.
A. Bar = 6.7 μm B. Bar = 3.3 μm

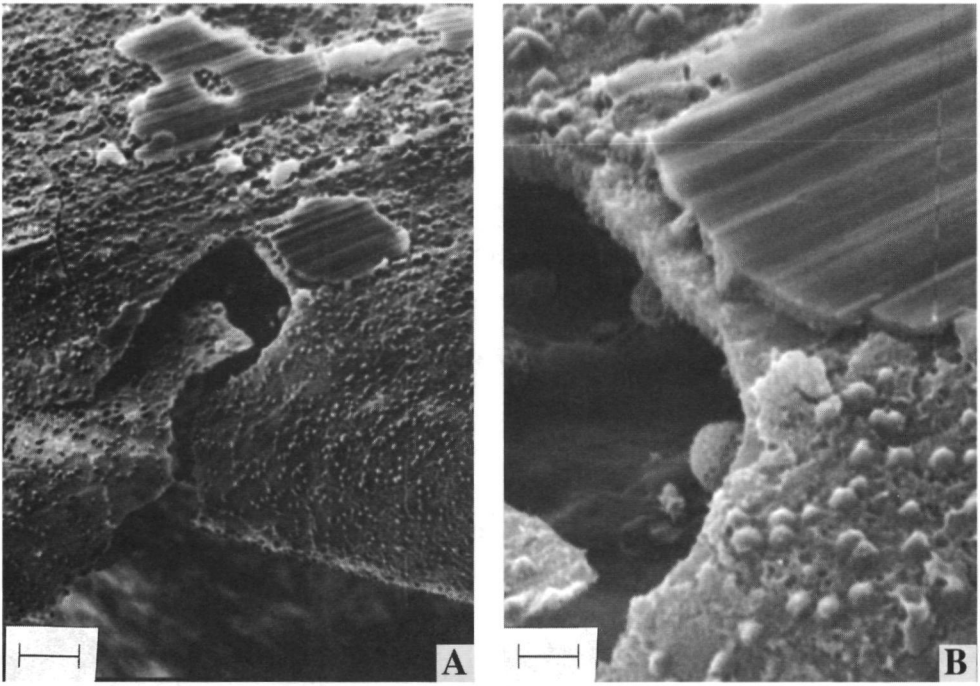


Figure 6 Scanning micrographs of RBM cells cultured for 18 days on a Ca-P-c coated specimen. A portion of the cell multilayer and ECM are raised, resulting in a good view on the a-fibrilaar mineralization foci in the ECM. Further, parts of the coating became detached from the titanium disc during the preparation and procedure and remained adhered to the ECM. It can be seen that the coating has followed perfectly the original titanium surface.
A. bar = 13 µm B. bar = 3 µm

Ca-P-c and HA-PS coatings. In contrast, the HA-PS/ht coating still did not show a sign of this kind of deposition structures (Figure 8).

2.4 DISCUSSION and CONCLUSIONS

The observations presented here again confirm the suitability of *in vitro* experimentation for studying cell-substrate interactions, enabling not only the evaluation of cellular behavior, but also its visualization on the electron microscopical level. Moreover, cell culture techniques have the advantage of offering a completely defined environment.

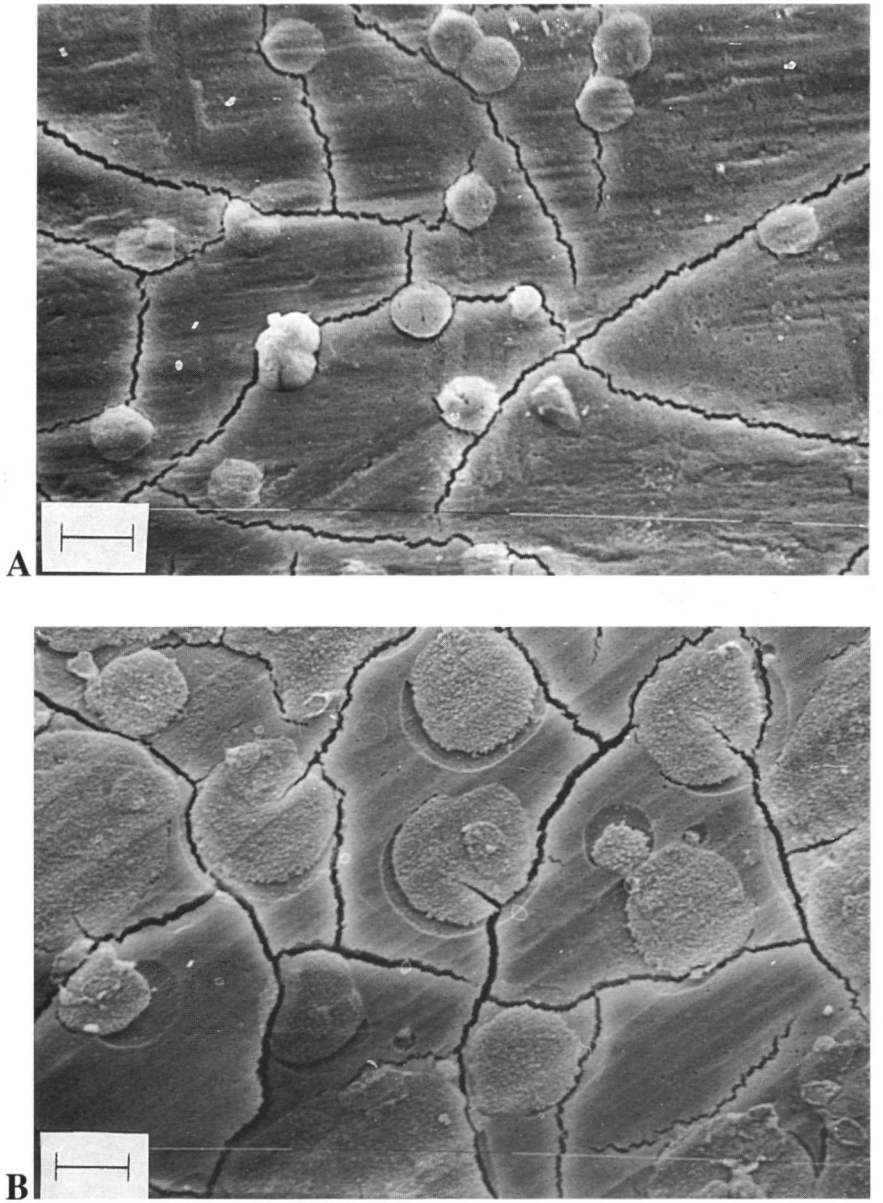


Figure 7 Scanning micrographs of the magnetron sputtered coatings soaked in fully supplemented culture medium for 6 days. All coatings show cracks, which are caused by the SEM preparation process. Spherulitic accretions are visible at the surface of both coatings (arrows).

A. Ca-P-a, bar = 3.2 µm

B. Ca-P-c, bar = 3.2 µm

The rat bone marrow cells attached and grew on all substrate surfaces. The question was raised whether this was correlated to the induction and formation of extracellular matrix. For example, no systematic signs of bone like tissue formation could be found on titanium substrates. Although, a definite reason for this effect of titanium is difficult to express, a possible explanation is given by Massas (1993). He cultured rat parietal bone cells on titanium and hydroxyapatite substrates. After an incubation period of 14 days the cells were equally proliferating on both materials. On the other hand, the alkaline phosphatase expression and parathyroid hormone response, characteristic of osteoblastic phenotype expression (Rodan, 1983), were higher in cultures grown on hydroxyapatite. Massas suggested that, since bone cell populations are heterogeneous, this increase is caused by the higher capacity of hydroxyapatite to support the proliferation and differentiation of osteogenic cells. A similar phenomenon can have occurred in our RBM cell cultures, resulting in more osteoblast-like cells together with ECM formation on the Ca-P coated specimens. This supposed influence of the substrate material upon osteoblast phenotype is further supported by Lian (1993). She demonstrated that osteoblast proliferation is related to the synthesis of ECM. The significance of these findings for the *in vivo* behavior of titanium and Ca-P coated implants will be clear and has already been confirmed in various ultrastructural studies (Linder, 1983; Steflik, 1992; de Bruijn 1993).

In contrast to these findings Davies (1990) reported ECM formation on titanium surfaces. A reason for this difference may be, that in these studies the cells were enzymatically released from the tissue culture flasks with 0.01% trypsin solution, while we used 0.25% solution. Since various proteolytic enzymes and concentrations can have a different damaging effect on morphology, growth rate and cellular activity (Escarot-Charrier, 1983; Schmidt, 1993; Callen, 1993), this might result in a loss of osteoblastic phenotype and ECM secretion.

Considering the attachment and proliferation assay, there are two other findings that need further explanation. First, the number of attached cells seeded on Ca-P-c coatings could not be measured by Coulter Counter. Examination revealed that detached coating particles obstructed the orifice of the counting tube. SEM inspection of the Ca-P-c coatings before their use in the cell studies, showed that these surfaces had a polycrystalline appearance. This grain structure is induced by internal stresses in the coating due the combination of heating of the titanium substrates during the magnetron sputter process and differences in thermal expansion coefficient to substrate and deposited ceramic coating. Consequently, after short incubation periods, when still no additional surface layers are deposited, this stressed Ca-P-c coating can easily be removed by rinsing procedures as used in the counting process.

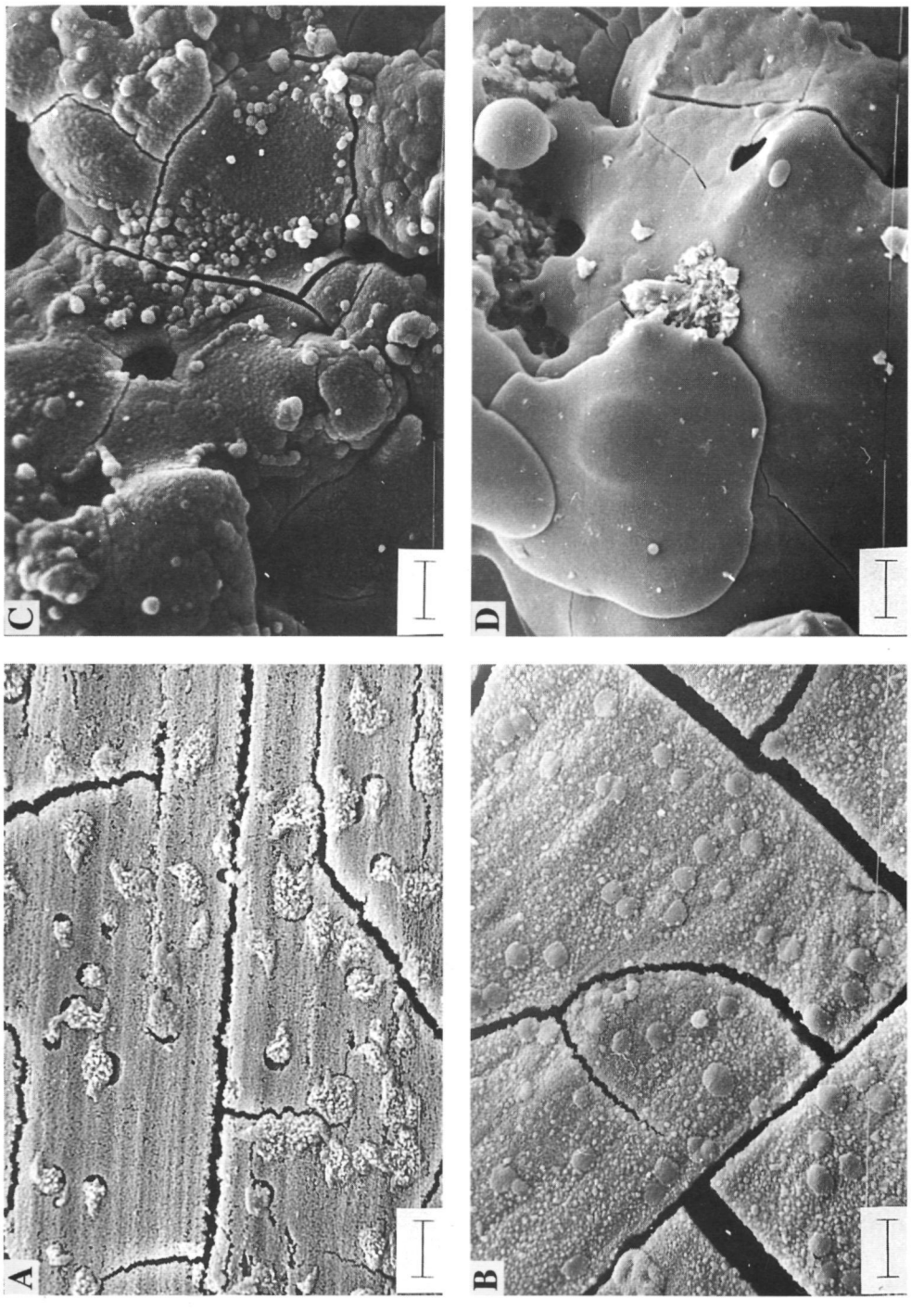

A
B
C
D

Figure 8 Scanning electron micrographs of all experimental coatings soaked in fully supplemented culture medium for 18 days. Compared with Figure 7, spherulite formation increased on the Ca-P-a, Ca-P-c and Ha-PS coatings. At the HA-PS/ht coating no sign of deposition structures can be recognized.

A. Ca-P-a, bar = 3.2 μm
B. Ca-P-c, bar = 3.2 μm
C. HA-PS, bar = 3.2 μm
D. HA-PS/ht, bar = 3.2 μm

A second issue is the incomplete removal of dissociated cells from the heat-treated plasma-spray coated substrates in the growth experiments. This observation justifies the conclusion that cellular proliferation has to be considered similar on all investigated surfaces. Besides, it emphasizes the importance of visual surface inspection in this kind of experiments.

Finally, the study to the influence of culture medium on the Ca-P coated discs revealed that small spherulites were deposited on Ca-P-c, Ca-P-a and HA-PS substrates, but not on the HA-PS/ht specimens. This surface behavior corresponds well with one of our physicochemical studies (Wolke, 1994), in which the dissolution and precipitation properties of amorphous, amorphous/crystalline and crystalline sputtered and plasma-sprayed coatings were determined after incubation in simulated body fluid (SBF). The Ca, P and Mg concentration of SBF is almost equal to human blood plasma (Li, 1992a). The coated specimens were soaked for 7 days in SBF and the fluid was changed daily. Spectrometric analysis showed, that for amorphous and amorphous/crystalline substrates the Ca and P concentration of SBF, after an initial increase, decreased until a constant concentration was obtained. HA-PS/ht did not change the ion concentration of SBF. SEM examination of the samples demonstrated the formation and growth of apatite nuclei on all surfaces, except for heat treated substrates. On basis of the spectrometric data and similar as described by Li (1992b, 1993) for silica gel, the start of this precipitation process can be attributed to a local supersaturation of both Ca and P caused by dissolution of these ions from the ceramic coating. The subsequent growth of the nuclei is controlled by the Ca and P ions already present in SBF. Tissue culture media contain also physiological concentrations of Ca, P and Mg ions. In addition, they are buffered at the same pH as SBF. Therefore, it can be assumed that, comparable to SBF, apatite induction processes will take place after immersion of Ca-P coated specimens in fully supplemented culture medium without cells. It will be evident that this apatite formation will influence the bonding properties and the final bone-substrate interface. Nevertheless, the full implication and clinical consequences of this phenomenon for sputter deposited Ca-P coatings have to be investigated further in transmission electron microscopical studies. Also, in these experiments, the required coating thickness to induce the most optimal apatite deposition has to be determined.

In summary, the experiments demonstrated that RBM cells cultured on magnetron sputtered Ca-P coatings stimulated the formation of ECM. Although the sputtered coatings detached in the short term attachment studies, no severe degradation of the coatings was observed in the prolonged culturing assays. In contrast even, sputtered coatings appeared to induce apatite formation. Based on these results, it can be concluded that magnetron sputtering is a promising method to manufacture bioactive ceramic coatings. If this can be confirmed *in vivo*, this coating process may be of advantage over the currently used techniques.

We thank Dr. A. Wetterwald from the Pathophysiological Institute of the University of Bern, Switzerland, who provided us with the antibody E11.

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CHAPTER 3

Evaluation of plasma-spray and magnetron sputter Ca-P coated implants; an *in vivo* experiment using rabbits

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3.1 INTRODUCTION

The resulting benefits (Geesink, 1987; Cook, 1988; Klein, 1991; Gottlander, 1992; Dhert, 1993) of plasma-spray HA-coated implants in dental and medical implantology may be summarized as: faster and greater bone adaptation; improved implant fixation; absence of intervening fibrous tissue; faster bone healing, which reduces the waiting period; and improved performance in poor bone quality.

On the other hand, concerns have been raised and some confusion remains regarding the viable use and prognosis of such coated implants.

These concerns deal with: (1) the substrate-to-coating fracture and fatigue strength properties, and (2) the biodegradation of the coating and what will happen at the implant-bone interface when the coating has disappeared, particularly in relation to the grit-blasting procedure required to obtain mechanical retention of the coating.

Therefore other deposition methods were developed to produce thinner (100 nm - 10 μm), more adherent and physiocochemically better-defined coatings. One of them, RF magnetron sputtering, has great promise and can possibly solve some of the concerns procured with the plasma spray techniques. The advantages of this procedure over the plasma spray technique, are: (1) more-adherent ceramic coatings can be deposited without preliminary grit-blasting of the implant surface, and (2) more-uniform ceramic coatings can be deposited, also on complex implant designs.

In view of this, experimental efforts in our laboratory have been directed to further development of this method for the deposition of thin Ca-P coatings on dental implants. Various physicochemical and biological experiments already have been performed. For example, preliminary research confirmed the apatite nature of the deposited coatings (Jansen, 1993; Wolke, 1994; van Dijk, 1995; van Dijk, 1996) and revealed the stabilility of sputtered coatings under cyclically loaded conditions (Wolke, 1995). In addition, cell-culture experiments demonstrated that the sputtered Ca-P coatings are biocompatible, and induce apatite formation (Hulshoff, 1995).

The purpose of the present study is to test in experimental animals whether magnetron-sputtered Ca-P coatings cause a similar or better bone behaviour than plasma-sprayed Ca-P coatings.

3.2 MATERIALS and METHODS

3.2.1 Implant materials and coating characteristics

Seventy-two specially designed cylindrical commercially pure titanium (cpTi) implants were made (Figure 1). All implants measured 8.0 mm in length.

Depending on the final coating procedure which was given to the implants, 36 of these implants had a diameter of 2.9 mm and the other 36 implants had a 3.0 mm diameter. The implants were provided with two rounded circular grooves (height, 2.0 mm; depth, 0.5 mm). Using the plasma-spray and magnetron-sputter technique the following coatings were deposited:

(1) plasma-spray coating (HA-PS),
(2) plasma-spray coating with additional heat treatment for 2 h. at 600 °C (HA-PS/ht),
(3) magnetron-sputter coating produced on a rotating substrate holder (Ca-P-a)
(4) magnetron-sputter coating produced on substrate holder in an indexed position (Ca-P-c).

For plasma spraying, commercially available hydroxylapatite powder was used with a particle distribution of 10-70 µm. Before coating deposition, implants were grit blasted with Al_2O_3 (Ra=4-5µm). Magnetron-sputter coating was performed as described earlier (Wolke, 1994; van Dijk, 1995; Hulshoff, 1995), using a commercially available unit (Edwards High Vacuum ESM 100 system). An HA plasma-sprayed copper disc served as a target for the sputter deposition. Before coating, the implants were mounted onto the rotating and water-cooled substrate holder. Coatings were applied on the as machined surface of the implants. The

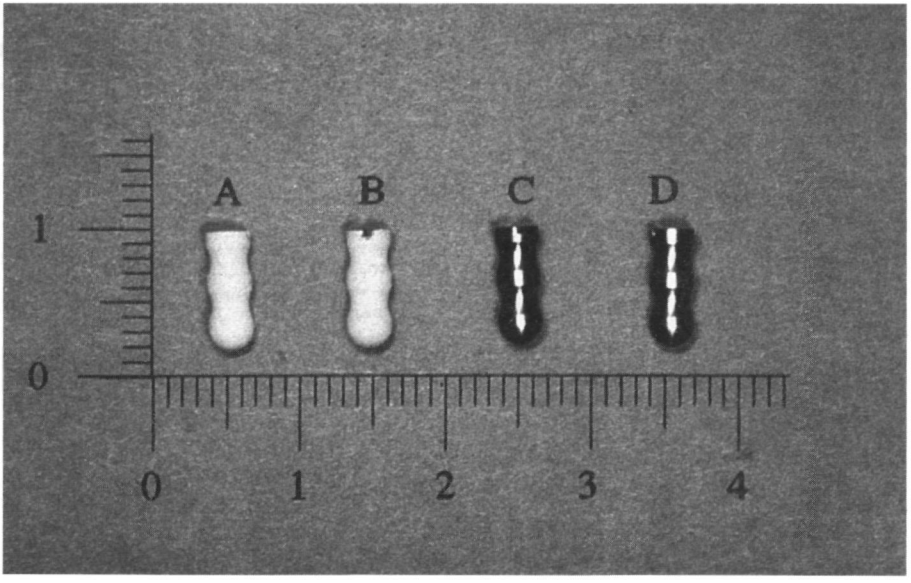


Figure 1 Implants with different Ca-P coatings.
A: HA-PS, B: HA-PS/ht, C: Ca-P-a, D: Ca-P-c

thickness of the plasma-sprayed coatings was about 50 μm, while the thickness of the magnetron sputtered coatings was 2.0-4.0 μm. Therefore the final diameter of all implants was similar (3.0 mm ± 0.004 mm). The chemical composition of all coatings was confirmed by X-ray diffraction (XRD), infrared spectroscopy and Rutherford Backscattering Spectrometry (RBS).

Before surgery, all implants were cleaned ultrasonically in 100 % ethanol. Subsequently, they were sterilized in an autoclave.

3.2.2 Experimental design and surgical procedure

Eighteen healthy female New Zealand White Rabbits, 3-4 months old, were used in this study. Surgery was performed under general anaesthesia by intravenous injection of Hypnorm (0.5 mL/kg) and atropine (0.5 mg/animal). After oro-tracheal intubation, anesthesia was maintained by ethrane 2-3% through a constant volume ventilator. To reduce the perioperative infection risk, prophylactic antibiotic (Terramicine®) was administered postoperatively by a subcutaneous injection.

The implants were inserted in both condyles of each femur. Therefore the animal was immobilized on its back and the hind legs were shaved, washed and disinfected with providone-iodine. A longitudinal incision was made on the medial side of the right and left femur. The medial as well as the lateral condyle could be exposed by this incision. At a distance of 0.5 cm from the femoral epifyse, a pilot hole was drilled and gradually widened to the final diameter of the implants. The bone preparation was performed at a low rotational drilling speed (500rpm) and continuous internal cooling. After press-fit insertion of the implants at the medial and lateral side, the skin was closed by intracutaneous sutures using Vicryl 3-0.

A total of 72 implants were placed; 18 HA-PS, 18 HA-PS/ht, 18 Ca-P-a and 18 Ca-P-c. Each animal received 4 implants, one at each lateral and medial side of the right and left femoral condyle. The position of the implants was confirmed by radiographs. For the localization of the implants a balanced split plot design was used to compensate for differences in bone quality and load characteristics between implantation sites.

Postoperatively the animals were placed in cages (five rabbits sharing one big cage, 1.33x1.10 m). They were provided with water and rabbit chow *ad libitum*, and were allowed to move unrestricted at all times.

The animals were sacrificed after 3, 6 and 9 weeks using an overdose of pentobarbitalsodium (Nembutal®) and the femoral condyles together with the implants were excised.

3.2.3 Histological procedures

Excess tissue of the excised femurs was removed immediately. At room temperature the specimens were fixed, in 4% formaldehyde buffered with PBS at pH 7.2-7.4, for one week. Then, tissue blocs were dehydrated by gradient series of ethanol. Subsequently, they were embedded in methylmethacrylate and finally polymerized at 37 °C. A modified diamond-blade microtome sawing technique (Klein, 1994) was used to prepare non-decalcified thin sections (approximately 10 μm) for examination by a light microscope. Sections were made in a horizontal plane, perpendicular to the long axis of the implant. Finally the sections were stained with methylene blue and basic fuchsin, and examined by transmittant light microscope.

3.2.4 Histological evaluation

Histological and histomorphometrical measurements were performed to evaluate the bone response. Histological evaluation consisted of thorough description of the observed tissue reaction. Histomorphometrical measurements were performed only on sections of the rabbits that were sacrificed after 6 and 9 weeks. For this histomorphometrical procedure a computer-based image analysis system (TCL-image) was used.

First the percentage of bone contact was determined. The amount of bone-implant contact was measured for the total implant perimeter. The percentage of bone contact was defined as the length of the interfacial area with direct bone-implant apposition.

Then the bone amount in circular regions around the implant was measured by placing the sections under a stereomicroscope, which was connected to a videocamera. With use of a frame grabber with 512x512 pixels, 8-bit grey-level images were captured. Two circular regions of interest (r.o.i.) were marked around the implant (Figure 2). One region was defined in direct contact with the implant, at a radial distance of 0.26 mm from the interface (circle A). The other region was determined at 0.61 mm from the implant (circle B). Finally, the amount of bone in the area confined by circle A and in an area called C (C = amount of bone inside circle A subtracted from the amount of bone inside circle B) were calculated by a computer based image analysis programme. The amount of bone was quantified as bone amount per $\mu m^2/10^3$.

For both quantitative bone evaluations three histological sections per implant, representative of the bone response, were randomly chosen. Presented results are based on the average of these three measurements. For each implantation period 6 implants of each coating type were evaluated.

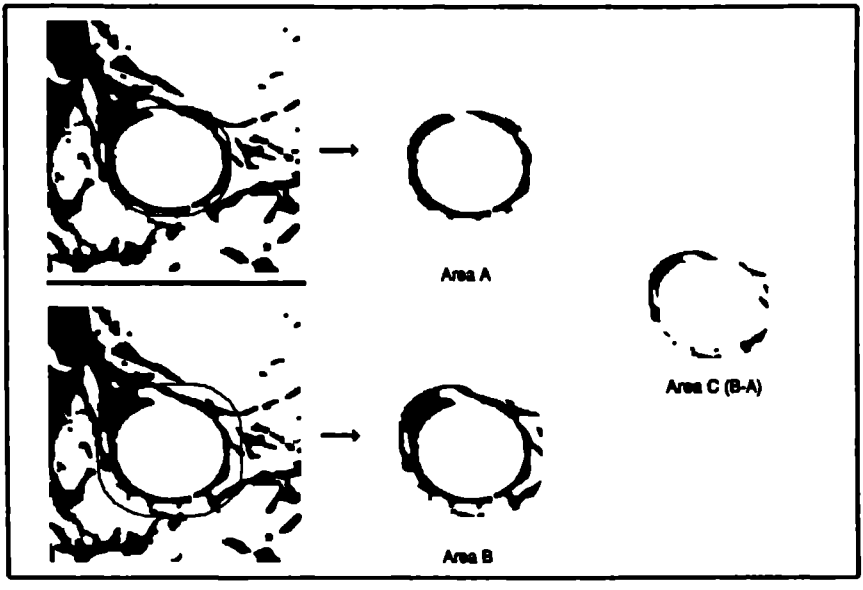


Figure 2 Illustration of the computer based image analysis measurements of bone amount.

3.3 RESULTS

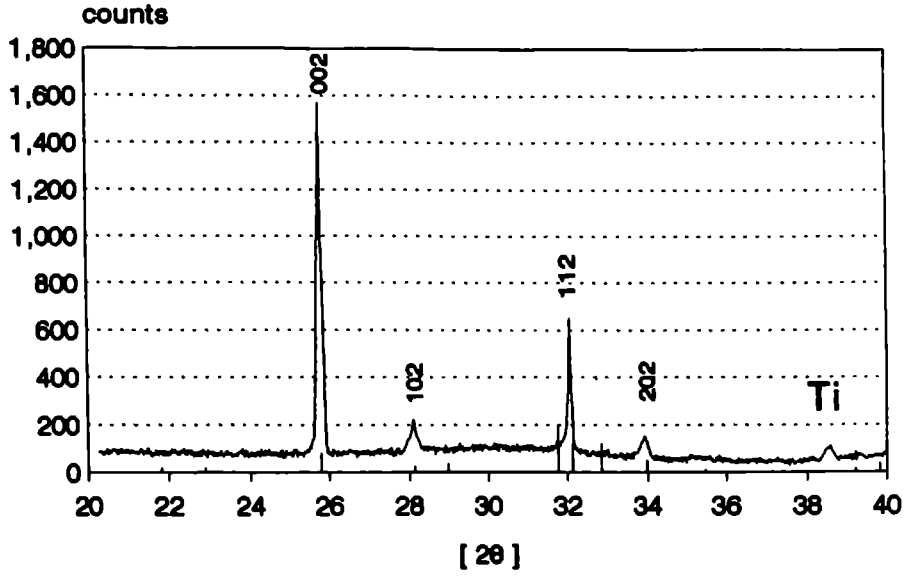
One of the rabbits, from the 3 week evaluation group, died of pneunomia. The other rabbits did not have any complications and appeared to be in good health during the experimental period. At sacrifice no clinical signs of inflammation or adverse tissue reaction could be seen around the implants. Radiographs, taken parallel to the long axis of the implant, showed that the implants were located in cortical as well as trabecular bone and the medular cavity of the femur.

3.3.1 Characterization of the Ca-P coatings

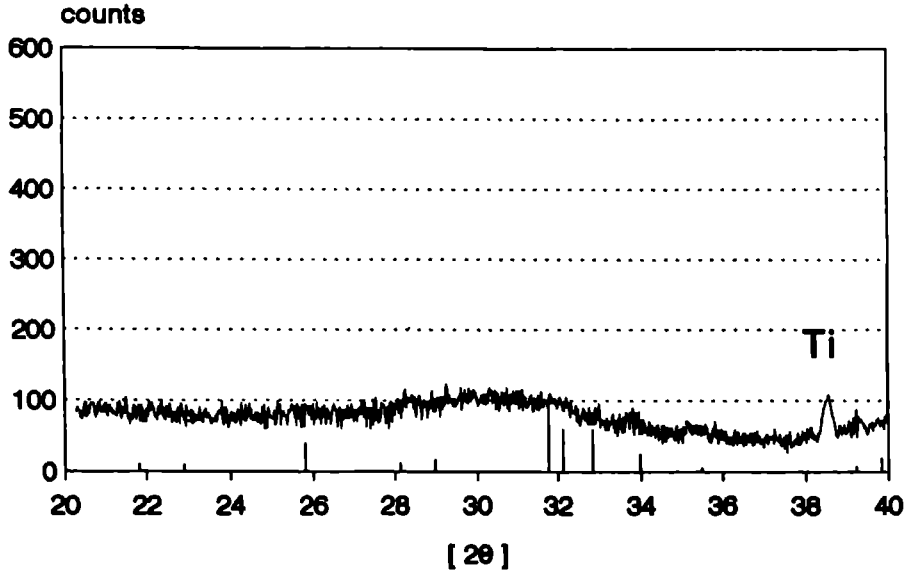
A complete description of the characteristics of the coatings has already been given elsewhere (Wolke, 1992; Wolke, 1994).

In summary, XRD patterns demonstrated that the HA-PS and HA-PS/ht coatings showed an amorphous/crystalline structure. After heat-treatment the crystallinity was increased (crystallinity HA-PS 60% vs. HA-PS/ht 65%). Analysis of the Ca-P-c and Ca-P-a diffractographs showed that the indexed procedure resulted in a crystalline Ca-P coating with an apatite structure. Coatings prepared with the rotating substrate holder showed an amorphous structure, without any specific reflection lines (Figure 3).

Infrared measurements of the plasma-sprayed samples showed absorption bands characteristic of P-O and O-H bonds. In the spectra of both sputtered coatings no



A



B

Figure 3 X-ray diffraction pattern of the magnetron sputtered coatings;
A: Ca-P-c magnetron-sputtered coating
B: Ca-P-a magnetron-sputtered coating

O-H bonds were detectable. These coatings showed a wide peak over the region from 2800 to 4000 cm^{-1} indicative for water absorption at the surface (Elliot, 1973).

RBS measurements of the sputtered coatings revealed for the Ca-P-c coating a Ca/P ratio of 2.8 and for the Ca-P-a coating a Ca/P ratio of 2.3 (Figure 4).

3.3.2 Light Microscopy.

As was observed in the radiographs, histological sections of the implants confirmed that the implants were positioned in cortical bone as well as trabecular bone and in the medullar cavity.

Three weeks

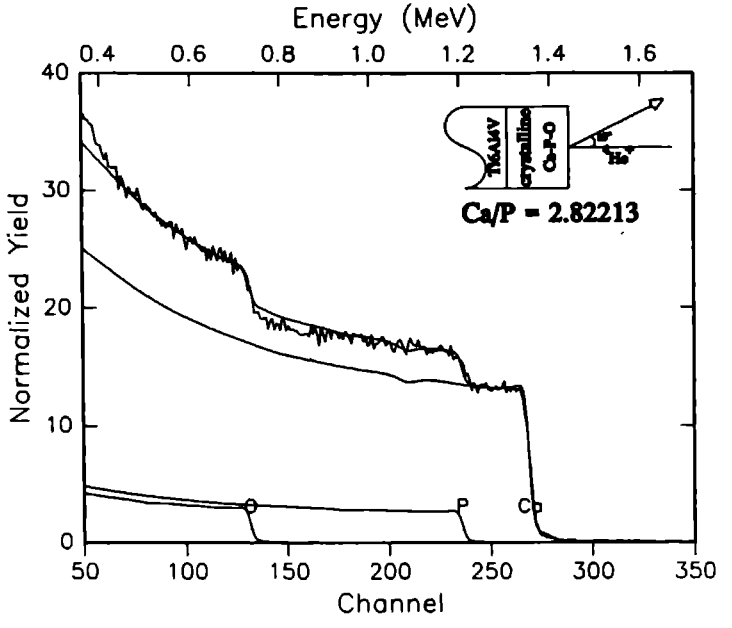
Three weeks after insertion around all implants a lattice of woven bone was formed, especially at those sites where the implants were positioned in the trabecular bone or medullar cavity (Figure 5). Occasionally, this callus had proliferated throughout the medulla. In some sections were noted limited areas with early signs of callus remodeling. All implants had a close bone-implant contact in the cortical area. At some sites where the implants were positioned without initial bone contact, a layer of osteoid covered the implant interface. For the HA-PS and HA-PS/ht coatings no signs of coating degradation were observed.

Six weeks

In these specimens the healing process had proceeded. In all histologic sections the callus was reduced in size, and had reoriented. The spaces in the lattice of the callus were filled with new bone. At some areas of the intramedullar bone-implant interface, new bone was deposited (Figure 6). On this deposited bone active osteoblasts and a layer of osteoid could be observed. This bone was in direct contact with the implant surface without intervening fibrous tissue layers. In some sections, the entire medullar surface of the implants was surrounded by newly formed bone, or a thin layer of osteoid covered the implant (Figure 7). Occasionally sections showed that bone was bridging the medullar cavity from the cortical walls to the implant surface. Furthermore sections made at the level of the circular grooves in the implants showed that the created gaps were completely filled and bridged with lamellar bone (Figure 8). The HA-PS and HA-PS/ht implants showed no substantial reduction in coating thickness.

Nine weeks

At nine weeks remodeling and compaction of the bone-implant interface was proceeding (Figure 9 and 10). No differences in bone formation between the various coatings could be observed. The plasma-spray coated implants still showed no signs of coating reduction. Only one of the HA-PS/ht coated implants showed



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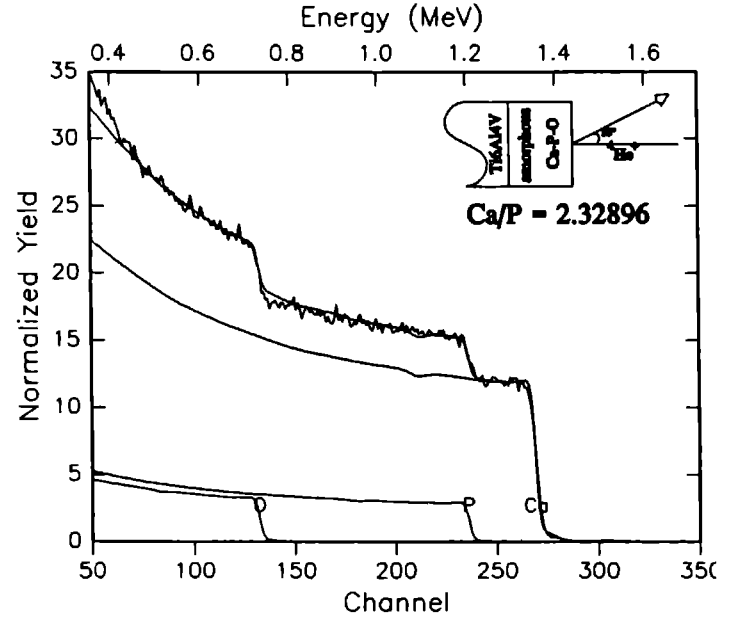

B

Figure 4 RBS measurements (^{4}He energy: 2.012 MeV and θ=10^O) of RF magnetron sputtered coatings;
A: Ca-P-c magnetron sputtered coating
B: Ca-P-a magnetron sputtered coating

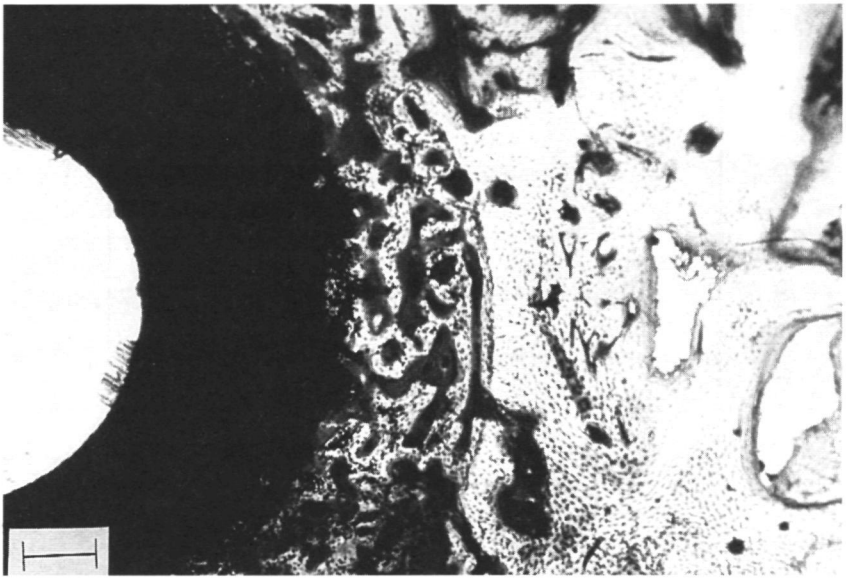

Figure 5 Light micrograph of a HA-PS/ht coated implant after 3 weeks of implantation. The original drilling hole can be recognized, because the slide is made at height of a circular groove of the implant. This gap has been filled with a lattice of woven bone. (Original magnification x10, bar = 300 μm.)

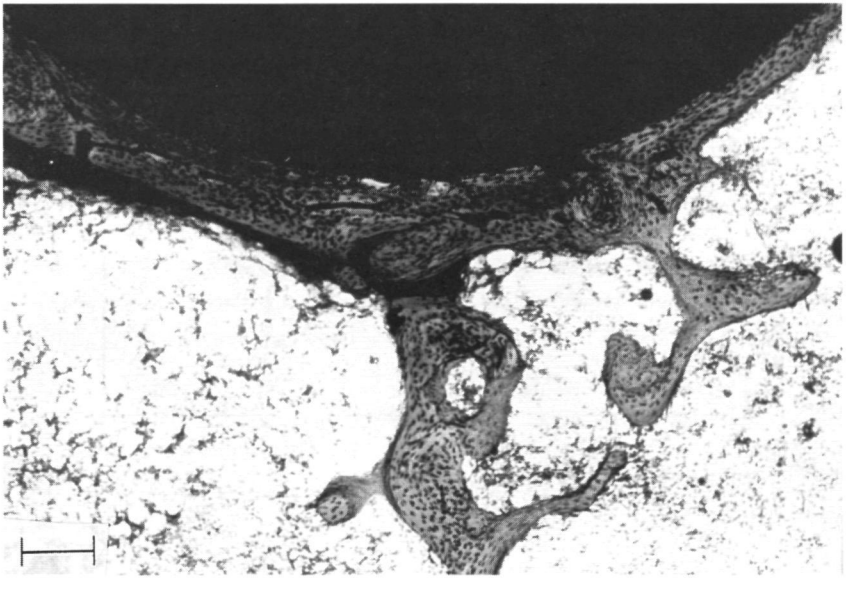

Figure 6 Light micrograph of a Ca-P-a coated implant after 6 weeks. New bone has been deposited in the medullar cavity. (Original magnification x10, bar = 300 μm.)

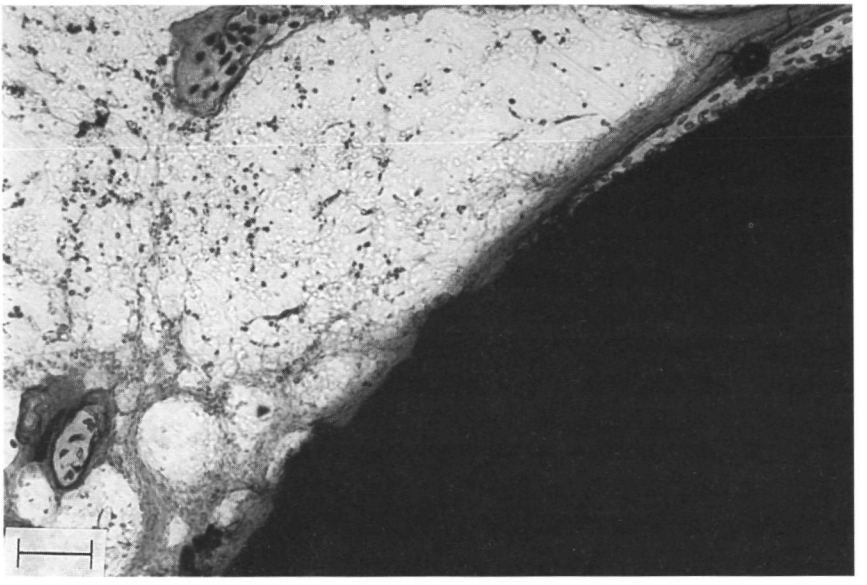

Figure 7 Histological appearence of a Ca-P-a coated implant after 6 weeks. The implant is partially positioned in the medullar cavity. In the upper right corner bone contact is shown. This bone is covered by a zone of osteoid (gray zone), which continues at the implant surface. (Original magnification x 32, bar = 94 μm.)

delamination of the coating. Bone tissue was found between the dislodged coating and implant surface (Figure 11).

3.3.3 Histomorphometrical measurements

Results are presented in Table 1. Statistical analysis of these data, using a one-way analysis of variance (ANOVA) and a multiple comparison test (Newman-Keuls), showed that no significant difference existed in bone contact between the various coatings ($P > 0.05$). In addition, no significant difference was demonstrated in bone contact between 6 and 9 weeks implantation time ($P > 0.05$).

Results of the bone-amount measurements are provided in Table 2. At 6 weeks ANOVA and Newman-Keuls revealed only a significant difference ($P < 0.001$) in bone amount in area C between Ca-P-c coatings and the other materials. At 9 weeks no significant differences existed in bone density in all areas for all materials ($P > 0.05$ and $P > 0.05$).

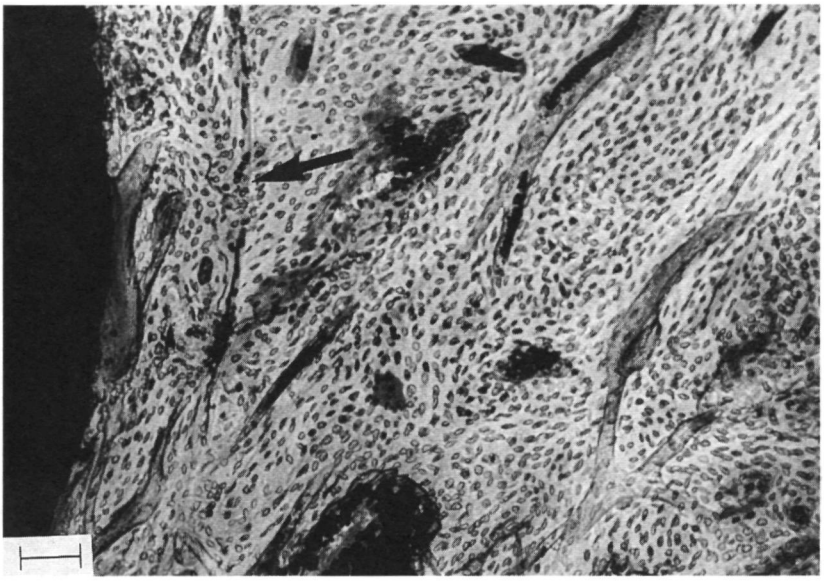

Figure 8 Histological section of a Ca-P-a coated implant after 6 weeks. The original drilling hole (arrow) can still be recognized. The healing process continues, lamellar compaction has started. (Original magnification x25, bar = 96 μm.)

3.4 DISCUSSION and CONCLUSIONS

This study is the first to present *in vivo* results of RF magnetron sputter coatings which are prepared, fully characterized, and finally tested in the same laboratory. Results revealed that sputtered Ca-P coatings induced the same behavior as plasma-sprayed coatings. At 6 weeks, even more bone was present around the Ca-P-c coatings compared with the other materials. Furthermore we observed that bone apposition to the various coatings did not decrease with time.

Although these results confirm our earlier *in vitro* studies (van Dijk, 1995; Hulshoff, 1995), they are in contrast with the findings of Steflik (1994). He evaluated HA plasma-spray coated, RF-sputter-coated and ion-beam-sputter-coated dental implants placed in the mandible of dogs. All implants were inserted using a so-called two-stage procedure, except for the RF-sputter-coated implants. These were one-stage implants. The evaluation periods were one and three months. Histomorphometrical analysis demonstrated that: (1) at 1 month postimplantation the percent of bone apposition to ion-beam-coated implants was higher compared to the other implants, (2) RF-sputter-coated implants showed significantly lower bone-contact percentages, and (3) the percent of bone-contact with ion-beam- and RF-sputter-coated implants dropped during the 3-month implantation period. The

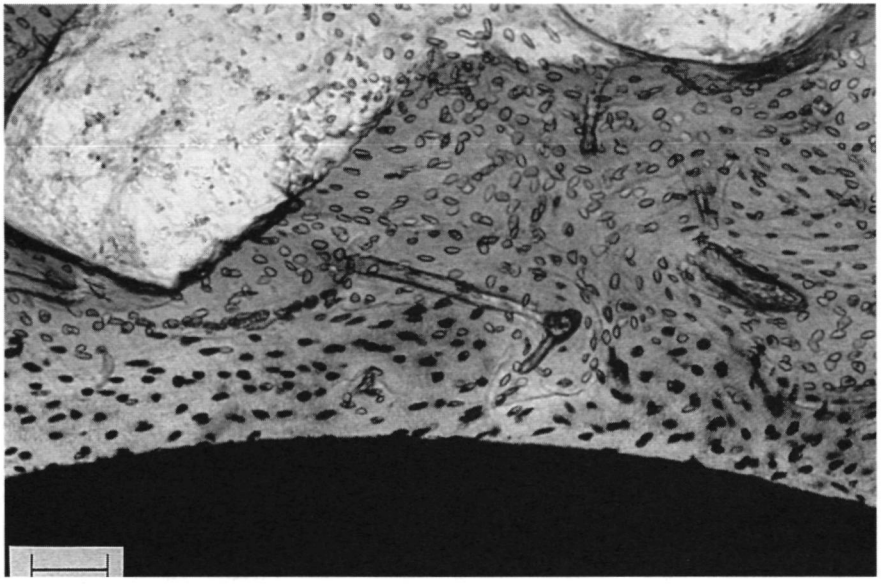

Figure 9 A Ca-P-a coated implant after 9 weeks from a light microscopcal view. Remodeling and compaction of the bone-implant interface has further proceeded. The bone is in close contact with the implant surface, without intervening fibrous tissue layers. (Original magnification x40, bar = 75 µm.)

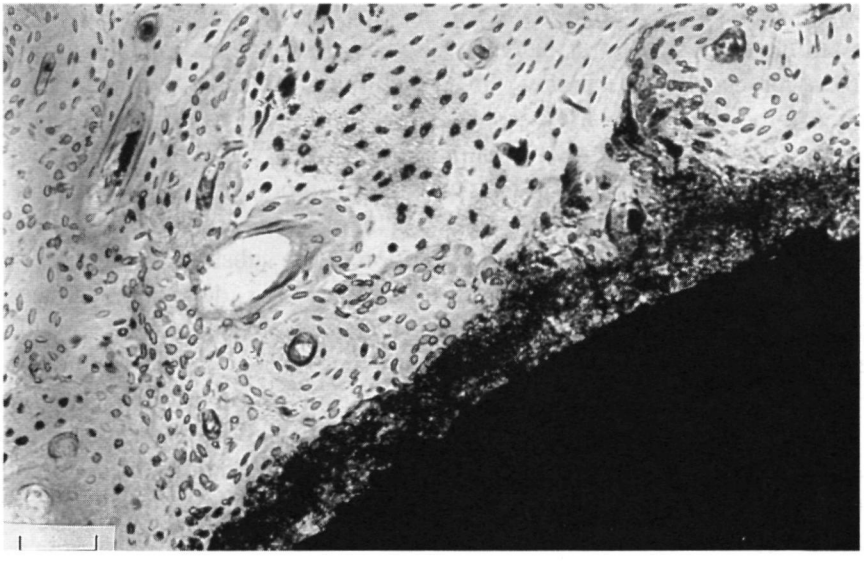

Figure 10 Light micrograph of a HA-PS coated implant after 9 weeks. Remodeling and compaction of the bone-implant interface has further proceeded. The plasma spray coating shows no sign of reduction in thickness. (Original magnification x10, bar = 300 µm.)

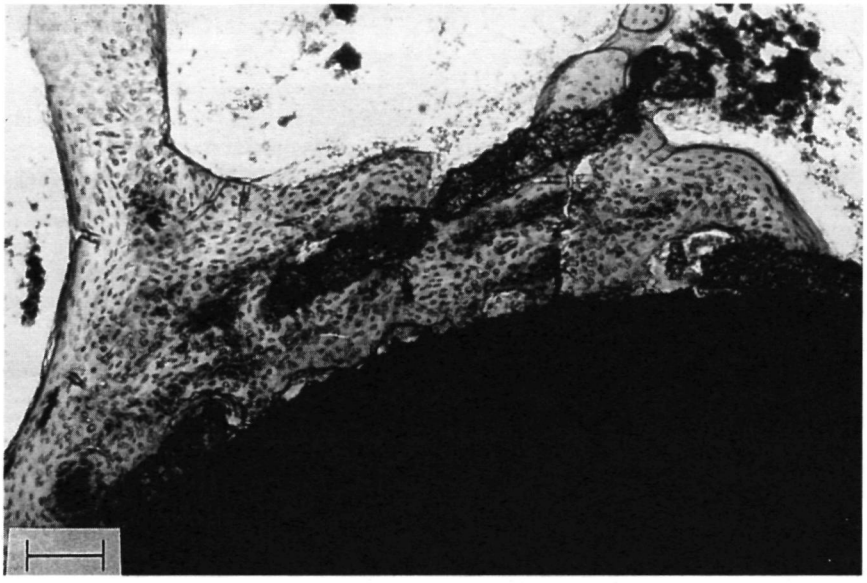

Figure 11 Detail of a HA-PS/ht coated implant after 9 weeks. The coating appeared to be detached from the implant. Bone tissue was found growing between the coating and the implant surface. (Original magnification x40, bar = 75 µm.)

following explanation can be given for the fact that our results do not corroborate these findings. First, Steflik subjected both types of sputter coating to a heat-treatment procedure to increase the crystallinity of these coatings. This heat-treatment procedure can vary the oxide properties of the titanium implant surface (Kasemo, 1991). Subsequently, when the coating dissolves during implantation, the newly formed bone will come in contact with this modified implant surface, which can result in a decreased bone response. In our study crystallinity of the RF coatings was obtained by indexing the position of the substrate holder. This makes an additional heat-treatment procedure after coating deposition unneccesary. Second, the lower bone adaptation to the RF coated implants can be caused by the different surgical procedure. One-stage implants are, in contrast to two-stage implants, directly exposed to the oral conditions. This makes their final behavior less comparable. Furthermore, since Steflik positioned all experimental implants of each type and implantation period in the left or right mandible of just one dog, it cannot be ruled out that the measured results are the interanimal differences of bone healing.

Also, some critical remarks have to be made on the thickness of the prepared histological sections. As demonstrated recently (Johansson, 1995), sections with a thickness of over 30 µm can lead to misleading interpretations of the true bone

contact. In addition, evaluation of an insufficient number of sections will result in a completely wrong conclusion about the bone reaction of an implant material. For example, in a previous study (Caulier, 1995), we showed that evaluation of three sections taken at different levels from the same implant revealed a significant variance in bone apposition. These two prerequisites for the correct estimation of bone contact, which were met in our study, also explain the large values of standard deviations in our histomorphometrical measurements.

With regard to the sputter coatings as used in our study another phenomenon has to be discussed. It is supposed that a Ca/P ratio of 1.67 together with an apatite structure is a prerequisite for a calcium phosphate ceramic implant to obtain a continuous interface with the surrounding bone (LeGeros, 1991; de Groot, 1984). However, despite their higher Ca/P ratio, the bone reaction to the sputter coatings was at least similar to the plasma-sprayed coatings. Such reactions have been observed earlier by Donath (1990). He inserted Ca-P ceramic implants with a Ca-P ratio ranging from 1.1 to 2 in the mandible of dogs. He found that the capacity of bone bonding increases even with the calcium content. Since the bioactive behavior of Ca-P ceramics is based on the dissolution of Ca^{2+}-ions and the subsequent precipitation of CO_3-apatite crystals (LeGeros, 1991), the enhancing effect of a high Ca content on the bone response is clear. Still, it has to be emphasized that the biological properties of Ca-P ceramics are, besides Ca/P ratio, also determined by phase composition and crystallinity. RF magnetron sputtering created Ca-P coatings similar to all „HA" materials, consisting of several Ca-P phases, i.e. HA, tricalcium phosphate and tetracalcium phosphate. In contrast to plasma-sprayed coatings, but similar to sintered ceramic materials, the crystallinity of sputter coatings is determined by atomic ordering and not by the size of the remaining powder particles. Therefore, the dissolution process will be identical to sintered ceramics and will not result in the release of harmful coating fragments. The biological advantage of RF magnetron-sputter coatings is that their major phase is HA in combination with an increased Ca content.

Table 1. Percentages of bone contact

	6 weeks		9 weeks	
COATING	% contact	stand.dev.	% contact	stand.dev.
HA-PS	63.1	10.8	71.3	11.1
HA-PS/ht	68.2	8.2	68.6	8.4
Ca-P-a	73.6	14.4	64.6	10.4
Ca-P-c	68.9	5.6	68.6	9.5

Table 2. Results of the bone amount measurements

	6 weeks				9 weeks			
COATING	area A	stand.dev.	areaC	stand.dev.	area A	stand.dev.	area C	stand.dev.
HA-PS	2326.67	2122.60	1859.83	417.11	2314.67	1182.14	1924.50	1034.11
HA-PS/ht	1997.50	1186.64	1755.67	551.73	1852.33	1254.32	1967.67	1169.02
Ca-P-a	2811.50	1687.79	1972.50	586.29	3242.33	2668.46	2396.17	1334.09
Ca-P-c	3736.60	1495.63	3822.80	1025.92	2540.83	912.50	2811.17	1665.90

Bone amount measurements are given in $\mu m^2/10^3$.

Another surprising finding was that the plasma-spray coatings showed few signs of coating reduction. This finding is in contrast with several other studies (Klein, 1991; Dhert, 1993; van Blitterswijk, 1993; Weinlaender, 1992; Søballe, 1993) in which plasma sprayed coatings were reduced significantly after 12 weeks of implantation. On the other hand it confirms our earlier studies with Ca-P plasma spray coated percutaneous implants (Jansen, 1990a,b). Reasons for this difference in observation can only be hypothesized. For example, the animal model used can influence the final coating behaviour. After insertion, the initial coating dissolution is followed by the precipitation of CO_3-apatite. It is suggested that this precipitated layer prevents further coating reduction (van Dijk, 1978). This process of dissolution and precipitation will be influenced by the wound-healing response of the animal model. In animals with a fast healing response, like rabbits and rats, the CO_3-apatite surface layer will be formed earlier or can be thicker than in animals with a slow healing response, like goats and dogs. Consequently, in fast-recovering animals less coating reduction will occur.

Degradation of the thin magnetron-sputter coatings could not be evaluated, since the histological sectioning technique used in combination with transmittant light microscopy makes it impossible to discriminate this layer from the underlying titanium. This can only be achieved by using transmission electron microscopy. Despite some favorable publications (Sennerby, 1992, 1993; Bjursten, 1990), which described techniques to obtain implant and bone tissue in one and the same ultrathin section, the preparation of TEM sections of solid metal and ceramic materials still is hampered by significant problems. Until a final solution has been found no reliable information can be gained about the closeness of the contact between the implant and bone tissue *in vivo*.

In conclusion, our study confirms that implants with magnetron sputtered Ca-P coatings situated inside the trabecular femoral bone of rabbits, show the same process of bone healing as plasma-sprayed Ca-P coated implants. However, with the magnetron-sputter procedure thin coatings (< 4 μm) can be produced, while plasma spraying results in coatings of at least 30 μm thick. Consequently,

accurately machined dental implants, with geometrically complex designs like screws, do not undergo dimensional changes because of the coating procedure. In addition, grit blasting to provide mechanical retention, as required for plasma spraying, is not required.

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CHAPTER 4

A histological and histomorphometrical evaluation of screw type calciumphosphate (Ca-P) coated implants; an *in vivo* experiment in maxillary cancellous bone of goats

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4.1 INTRODUCTION

Considering the concern which has been raised about the viable use and long term prognosis of plasma-sprayed calcium phosphate (Ca-P) coated implants (Kangasniemi, 1994, Dalton, 1995), experimental efforts in our laboratory have been directed to the development of a RF magnetron sputter technique for the deposition of Ca-P coatings (Jansen, 1993a; Wolke, 1994; van Dijk, 1995, 1996). In earlier studies we demonstrated that previous roughening of an implant, like for plasma-spraying, is not required to produce an adhesive coating (Jansen, 1993a). In addition, physicochemical analysis confirmed the apatite nature of the applied films (Jansen, 1993a; Wolke, 1994; van Dijk, 1995, 1996). Cell culture experiments demonstrated that the sputtered coatings were biocompatible and induced apatite formation (Hulshoff, 1995). Finally, in an *in vivo* rabbit experiment, where sputtered and plasma-spray coated implants were installed in the trabecular bone of the femoral condyles, we found no difference in bone healing response between these two types of Ca-P coatings (Hulshoff, 1996).

Despite these favorable results we know that the final bone response to an implant is also determined by the quality of the surrounding bone. For example, it is supposed that bone of low density, as found in the maxilla, can delay the bone repair during the healing phase. To investigate this influence, we developed a goat animal model to install oral implants into the low density trabecular bone of the maxilla. Using this model, Caulier *et al.* observed a significantly improved bone response to plasma-spray Ca-P coated implants (Caulier, 1997).

In view of the above mentioned, the aim of the present investigation was to study the biological behaviour of plasma spray and magnetron sputter Ca-P coatings in the low density trabecular bone of the goat maxilla.

4.2 MATERIALS and METHODS

4.2.1 Implant materials and coating characteristics

Forty eight commercially pure titanium implants (cpTi) with a tapered, conical screw design (BioComp® Industries, The Netherlands) were used (Figure 1). All implants measured 10.0 mm in length. Depending on the final coating procedure which was given to the implants, 24 of these implants had a diameter of 3.9 mm, and the other 24 implants had a 4.0 mm diameter. This diameter was measured at the upper coronal neck of the implant. Using the plasma-spray and magnetron sputter technique the following coatings were deposited:

1. twelve 3.9 mm implants were provided with a hydroxyapatite plasma-spray coating (HA-PS) of approximately 50 μm thick, using spray dried powder with a

mean particle size of 32.5 µm and consisting of 99% HA (CAM 2, CAM Implants B.V., Leiden, The Netherlands).

2. the other 12 implants with a 3.9 mm diameter were provided with an experimental bilayered Ca-P coating (CAM Implants B.V., Leiden, The Netherlands) of approximately 60 µm thick (FA/HA-PS). First the implants were coated with a 30 µm thick fluorapatite layer. This layer was subsequently covered with a 30 µm thick hydroxy-apatite coating. The fluorapatite powder had a mean particle distribution of 36.9 µm and a composition of > 99% FA. For the HA layer the same powder was used as for the HA-PS implants.

3. twelve of the 4.0 mm implants were provided with a magnetron sputter coating (Ca-P-a) with a thickness of approximately 2.0 µm.

In addition twelve 4.0 mm implants (cpTi) were left uncoated.

To obtain adhesion of the plasma spray coatings, implants were grit blasted with Al_2O_3 to a surface roughness of R_a=4-5µm.

Magnetron-sputter coating was performed as described earlier (Jansen, 1993a; Wolke, 1994; van Dijk, 1995, 1996), using a commercially available unit (Edwards High Vacuum ESM 100 system). An HA plasma sprayed copper disc served as a target for the sputter deposition. Before coating, the implants were mounted onto the rotating and water-cooled substrate holder. Coatings were applied on the as machined surface of the implants.

The chemical composition of coatings was confirmed by X-ray diffraction (XRD), Fourier Transform Infrared Spectroscopy (FTIR) and Rutherford Backscattering Spectrometry (RBS).

XRD (Figure 2) of the plasma sprayed FA part of the dual coating revealed >98% crystallinity, and the HA part of this coating was 60% crystalline. For the plasma sprayed HA coating, 64% crystallinity was measured. FTIR showed partial dehydroxylation for both, HA-PS and the HA part of the FA/HA-PS coatings.

The XRD pattern (Figure 2) of the Ca-P-a coating showed an amorphous structure without any specific reflection lines. Analysis of the FTIR spectra of the Ca-P-a coating showed a broad region, which can be attributed to the internal vibrations of phosphate-groups. This is indicative for the formation of amorphous calcium phosphate phases. Also a large H_2O region was shown. RBS measurements of this coating revealed a Ca/P ratio of 2.2.

Before surgery, all implants were cleaned ultrasonically in 100% ethanol. Subsequently, they were sterilized in a steam autoclave.

4.2.2 Experimental design and surgical procedure

Twelve healthy, mature (2-4 years of age), female Saanen goats, weighing about 60 kg were used in this study. Prior to surgery, blood samples of the goats were taken to ensure that the animals were CAE/CL arthritis-free. The animals were housed in a stable.

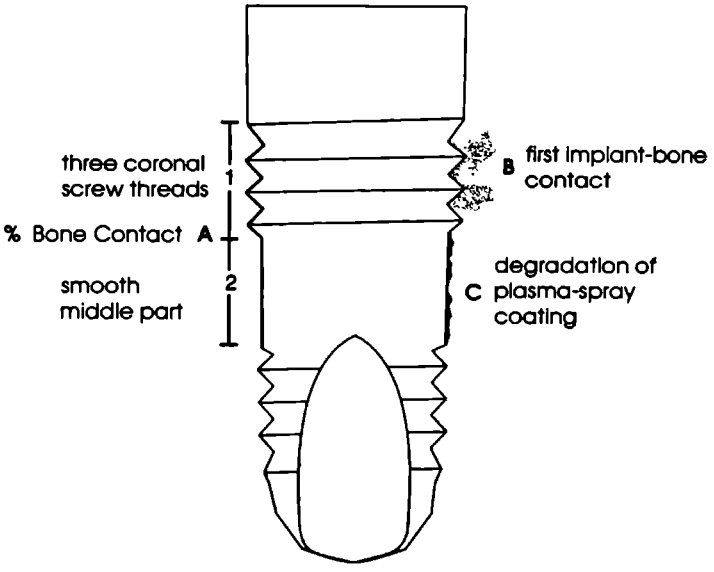

Figure 1 Schematic drawing of the tapered conical screw type implant
Areas of interest (A, B, C) for histomorphometrical evaluation are indicated.

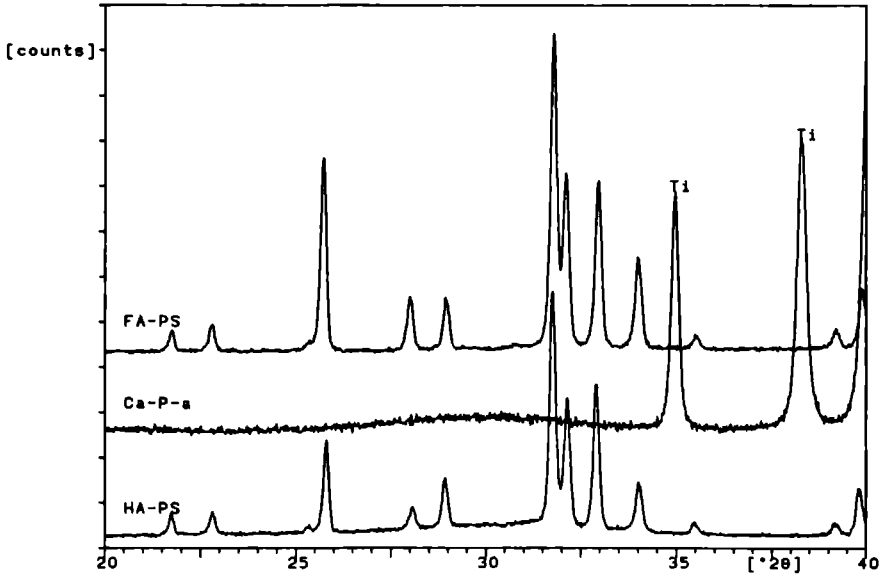

Figure 2 XRD patterns of the different Ca-P coatings with the 2 θ in degrees on the x-axis, and the relative counts on the y-axis.

From each of the twelve goats, the first and second maxillary premolars were bilaterally extracted. The goats were immobilized on their backs and anaesthesia was induced by an intravenous injection of Thiopental® and maintained by ethrane 2-3%, administered through an orotracheal tube. The mouth of the goats and the skin around the mouth were disinfected with Betadine-iodine. After careful extraction, the wounds were closed using resorbable vicryl 2-0 sutures.

Four months after tooth removal, again under general anaesthesia, implants were inserted in the edentulous part of the maxillary premolar regions. A longitudinal incision was made on the palatal side of the alveolar ridge and a full thickness mucoperiosteal flap was prepared to both the buccal and palatal side of the alveolar ridge. After exposure of the alveolar ridge, two 1.6 mm pilot holes were drilled. The distance between the two holes was approximately 1 cm. These holes were gradually widened with drills to the final diameter (4 mm) of the implants. The bone preparation was performed with a very gentle surgical technique using low rotational speeds (max. 450 rpm) and continuous internal and external cooling. After implant insertion, the wounds were closed using resorbable sutures (vicryl 2-0). To reduce the risc of peri-operative infection, a prophylactic antibiotic (Albipen®) was administered for three days. Each goat received 4 implants; 2 in the left and 2 in the right maxillary alveolar ridge. The implants were inserted following a balanced split plot design. Three and six months after implantation a group of 6 animals was sacrificed by an overdose of Nembutal®.

4.2.3 Histological procedure

After sacrificing the animals, the maxillae were excised *en bloc*. These were divided into two smaller tissue blocks, a left and a right part; each containing two implants. X-rays were taken of these blocks to determine maintenance or loss of the implants. Thereafter they were fixed in 4% buffered formalin solution. Following dehydration by series of ethanol, the tissue blocks were embedded in methylmetacrylate. After polymerization nondecalcified thin (10 µm) sections were prepared in the buccopalatal direction parallel to the long axis of the implant, using a modified sawing microtome (FMTA, The Netherlands) (van der Lubbe, 1988; Klein, 1994). The sections were stained with methylene blue and basic fuchsin for light microscopical evaluation.

4.2.4 Histological and histomorphometrical evaluation

A Zeiss light microscope was used for the histological evaluation. In addition, image analysis techniques (TCL-image) were used for histomorphometrical evaluation.

The following quantitative parameters were assesed:

A. *percentage of bone contact at the interface*

Measurements were performed: 1. along three coronal screw threads, and 2. at the smooth middle part of the Biocomp® implant (Figure 1). The amount of bone contact was defined as the percentage of implant length at which there is direct bone-to-implant contact without intervening soft tissue layers.

All quantitative measurements were performed for 3 different sections per implant. Measurements were done as well for the buccal as the palatinal site. Presented results are based on the average of these three measurements.

B. *first implant-bone contact*

Measured as the distance from the top of the implant to the first bone contact. The heigher this value (minimum 0 mm, maximum 10 mm), the lower the first bone contact (Figure 1).

Similar to A, the measurements were based on three sections per implant.

C. *degradation of the plasma spray coatings*

Measurements were performed at the smooth middle part of the implant (Figure 1). Images were projected with a total magnification of 125 x on a color monitor, using a color camera attached to the light microscope. Subsequently 14 scan lines were projected over the video picture, perpendicular to the implant surface, and the spacing between the coating boundaries was calculated. This procedure resulted in a distant count of 168 for each coating type.

4.3 RESULTS

During the test period the experimental animals remained in good health; no complications were observed. X-rays of the tissue blocs at sacrifice, showed loss of 3 Ca-P-a coated and 2 cpTi implants. Two implants were lost in the 3-months group; 1 Ca-P-a and 1 cpTi implant. Three implants were lost in the 6 month group; 2 Ca-P-a and 1 cpTi implants (Table 1). The remaining 43 implants healed without any clinical signs of inflammation. A Chi-square test revealed no significant difference in loss or maintenance between the Ca-P coated and non-coated implants, neither for the 3-months, nor for the 6-months group.

4.3.1 Light microsopical evaluation

Figures 3a and 3b show that the trabecular bone density in the goat maxilla is very low. The implants were positioned between the buccal cortical bone plate and the maxillary sinus. Examination of the histological sections revealed that 5 implants were partially situated inside the maxillary sinus. This did not result in an

TABLE 1:
Loss and maintenance of the different implant materials for the different implantation periods

	3 months		6 months	
Material	maintenace	lost	maintenance	lost
FA/HA-PS	6	0	6	0
HA-PS	6	0	6	0
Ca-P-a	5	1	4	2
cpTi	5	1	5	1
Total	22	2	21	3

adverse tissue reaction. The bone reaction to both types of plasma spray coated implants was relatively uniform (Figures 4a and 4b). At the interfaces of these implants large areas showed intimate contact with mature bone, while other parts showed a newly formed layer of bone. In addition, at 3 months parts of the implant interface were covered with a layer of osteoid. At 6 months more newly formed bone was recognized.

The cpTi and the Ca-P-a coated implants showed a very limited amount of bone contact. Most implants were almost completely surrounded by a fibrous tissue capsule (Figures 5a and 5b). At 3 months bone was clearly separated from the implant. At 6 months bone grew closer to the implant interface. Also after 6 months of implantation some implants showed bone contact at the apex of the implant.

Both the HA-PS and FA/HA-PS coated implants revealed signs of degradation (Figures 6a and 6b). This reduction was not uniform. For both, at some places the entire coating thickness was maintained, while at other sites no coating or only a thin layer was left. Absence of parts of these plasma spray coatings did not restrain direct bone contact: intimate bone contact could be recognized at these sites. A complete absence of plasma spray coating was more often seen for the HA-PS coating than for the FA/HA-PS coating. It appeared as if the HA-PS coating diffused inside its surrounding tissue, especially when it was not a mature type of bone. The remained part of coating of the FA/HA-PS coating showed a more compact appearence.

4.3.2 Histomorphometrical evaluation

In Table 2 results of the bone contact measurements are presented. As indicated by these results, minimal amount of bone contact was measured for cpTi and Ca-P-a implants.

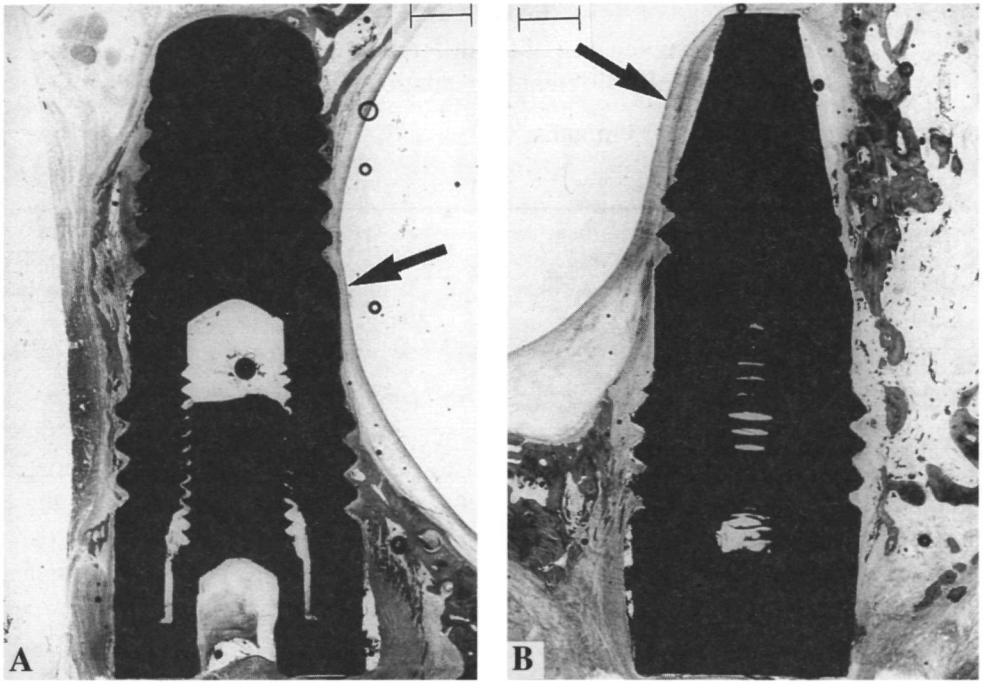

Figure 3 Light micrograph of (A) an HA coated implant after 6 months of implantation, and (B) a Ca-P-a coated implant after 6 months of implantation. Low density of the trabecular bone of the goat maxilla can be noticed. Close proximity (A) to the maxillary sinus, and an implant position inside the sinus (B) is shown (arrows).
(Original magnification x 2.88. Bar = 1.043 mm)

TABLE 2

Percentages of bone contact for the various implants for both implantation periods

	3 months			
	screw threads	std	smooth part	std
FA/HA-PS	53.9	13.4	67.2	26.5
HA-PS	32.7	24.5	75.5	21.0
Ca-P-a	0.0	0.0	0.0	0.0
cpTi	3.9	8.8	4.0	8.9
	6 months			
	screw threads	std	smooth part	std
FA/HA-PS	59.8	22.5	41.9	16.1
HA-PS	50.3	12.9	46.9	13.7
Ca-P-a	4.0	5.2	5.1	3.5
cpTi	4.2	5.1	5.1	8.1

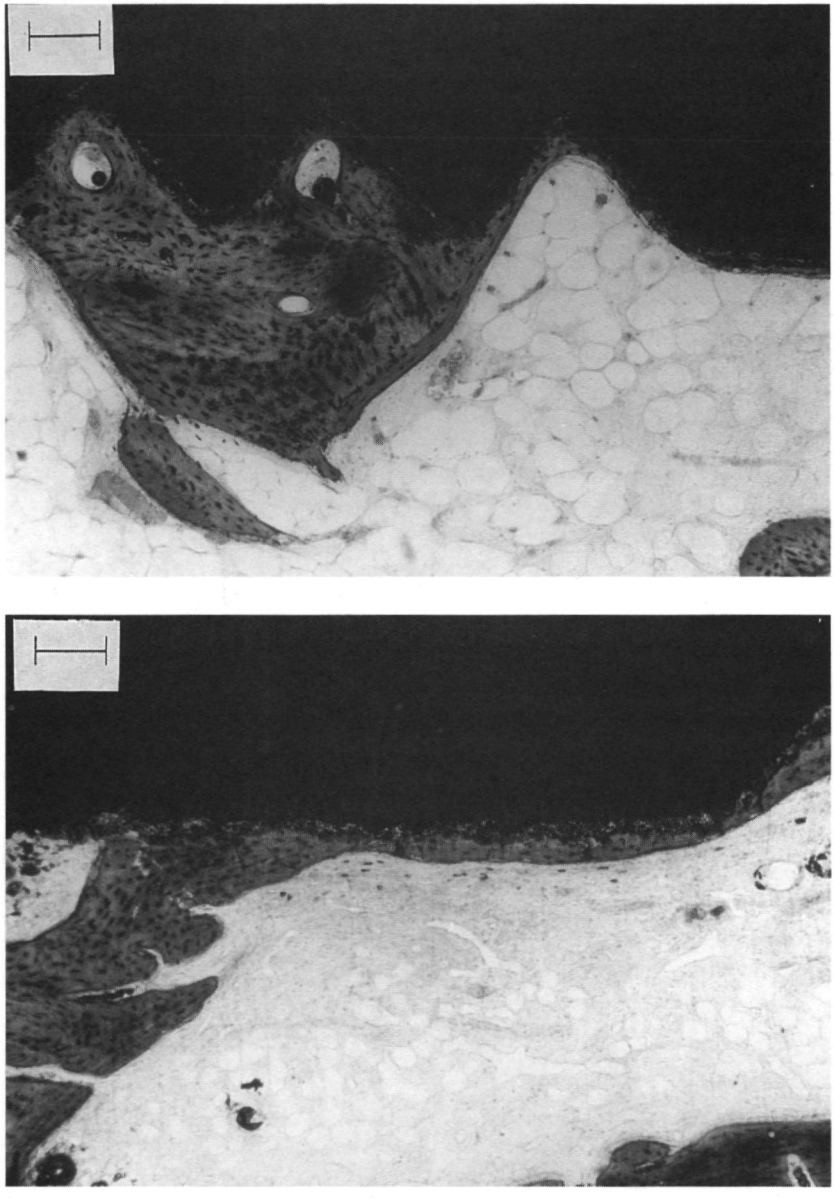

Figure 4 Light micrograph of (A) a HA-PS coated implant, and (B) a FA/HA-PS coated implant, both after 6 months of implantation. Good bone implant contact can be observed. (Original magnification x 16. Bar = 187 μm.)

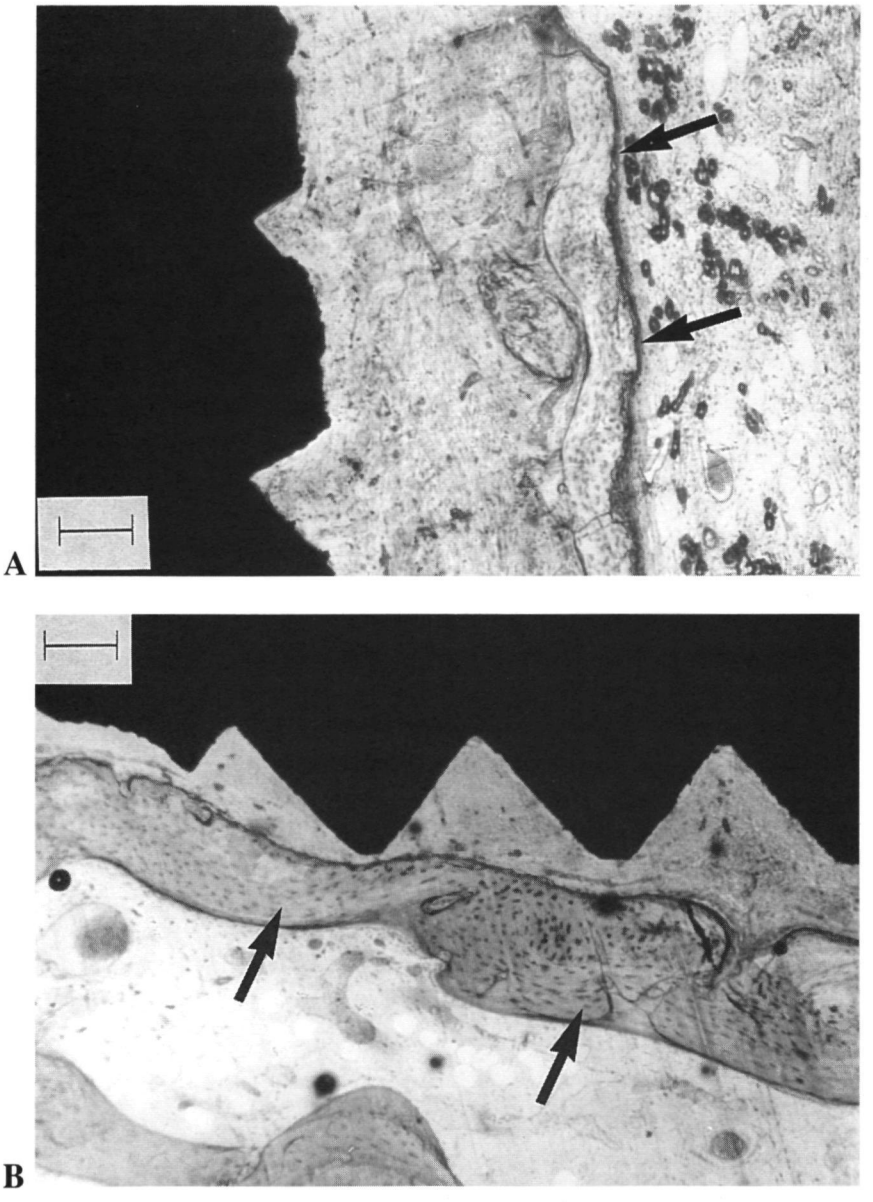

Figure 5 Histological appearance of (A) a Ca-P-a coated implant and (B) a cpTi implant, both after 3 months. A fibrous layer of tissue interposes between bone (arrows) and implant. (Original magnification x 16. Bar=187 µm.)

Statistical testing of the results, using a one way ANOVA and Tukey multiple comparison procedure revealed:

* A significant difference ($p < 0.001$) in bone contact between both types of plasma spray coated implants and the two other implant surfaces (Ca-P-a and cpTi).
* No significant difference ($p < 0.05$) in bone contact existed between FA/HA-PS and HA-PS at both implantation periods.
* For all implant types no significant differences ($p < 0.05$) between 3 and 6 months of implantation.

Figure 7 shows results of the first implant-bone contact measurements. Measurements for the Ca-P-a and cpTi were not involved in the bardiagram, since at both implantation periods all of these maintained implants were almost completely covered by fibrous tissue (see Table 2). Statistical testing revealed that no significant difference existed ($p < 0.05$) in the level of first implant-bone contact between both plasma spray coated implants for 3 and 6 months of implantation. In addition no significant difference ($p < 0.05$) was found between the implantation periods.

Results of the measurements of degradation of the plasma spray coatings are presented in Table 3.

4.4 DISCUSSION

The aim of the study was to investigate the behaviour of plasma-spray and RF magnetron-sputter Ca-P coatings in the low density trabecular bone of the goat maxilla.

The results revealed that plasma-spray coated implants showed more bone contact compared to non-coated and magnetron sputter coated implants after 3 and 6 months of implantation. These findings confirm the results of other studies (Caulier, 1995, 1997; Jansen, 1991; Dhert, 1993) with plasma-spray Ca-P coated and non-coated implants. In addition, the data with the non-coated as machined Ti implants correspond with the earlier studies of Caulier *et al.* (1995, 1997). She also observed a very low percentage of bone contact to cylindrical screw shaped oral implants inserted in the goat maxilla.

In contrast with previous *in vitro* and *in vivo* observations (Hulshoff, 1995, 1996), our study failed to proof the beneficial effect of RF magnetron sputter Ca-P coatings on the bone healing response. The inferior bone reaction, as found in the present study, can be due to the used animal model and the amorphous structure of the sputter coatings. The implants were inserted in a host bed of extremely low mineralization and density as compared to our earlier experiment in the trabecular

bone of rabbits. After insertion in bone tissue the bioactivity of Ca-P ceramics is based on their ability to become coated with a layer of bone mineral (carbonate apatite) (Jarcho, 1977, 1981). Normal remodeling of this at the surface of the ceramic deposited, bone layer occurs. The Ca-P ceramic, when it is dense sintered does not penetrate in the remodeling process nor is it resorbed by other processes. On the other hand, Ca-P coatings on metallic substrates are subject to degradation, resulting in reduction of thickness (Caulier, 1995; Jansen, 1993a,b,c, Klein, 1991, 1994b, Dhert, 1991). For thin sputter-deposited coatings, the *in vitro* dissolution behaviour is determined by the degree of the coating s crystallinity (Wolke, 1994). It can be supposed that this dissolution process will be influenced by the wound healing response of the animal model. Species with a fast healing response (rats and rabbits) will show earlier formation of the carbonate apatite surface layer, than animals with a slow healing response (goats and dogs). This hypothesis is supported by a recent study, in which the surface features and dissolution properties of various amorphous and crystalline Ca-P sputter coatings were determined after subcutaneous implantation in rabbits (Wolke, 1996). All amorphous coatings dissolved within four weeks of implantation. Crystalline coatings showed deposition of a granular precipitate consisting of Ca and P with a Ca/P ratio of 1.0 to 1.75. This precipitate grew in thickness until 4 weeks of implantation, while the coatings were still maintained. Consequently, we suppose that complete dissolution of the amorphous coatings, as used in the current study, occurred, before it had the chance to influence the healing response. In addition, this explains why no significant difference in bone contact existed between the sputter-coated and non-coated Ti implants.

Another remark has to be made about the difference in bone contact between both plasma-spray coated and the other implants. The plasma-spray coatings show a rough surface appearance. This may be an additional factor in the favorable bone response. This is confirmed by the results of other investigators (Buser, 1991; Wennerberg, 1995), who described a stimulation of bone-implant contact by surface roughening compared to machine-prepared implants.

The present study showed no significant difference in bone reaction to the various implant surfaces between the 3 and 6 months installation periods. Therefore the question arises whether an intervening healing period of 6 months is necessary before loading an oral implant, even when they are installed in bone of low density. Although this type of bone, according to Lekholm and Zarb (1985), can be defined as bone quality IV, it is no "diseased" bone with a lower healing capability. Evidently, bone healing after implant installation cannot be related to bone quality.

Considering the bone contact measurements, it has to be noticed that also the implant design can have increased the initial bone formation. Studies of Pilliar *et*

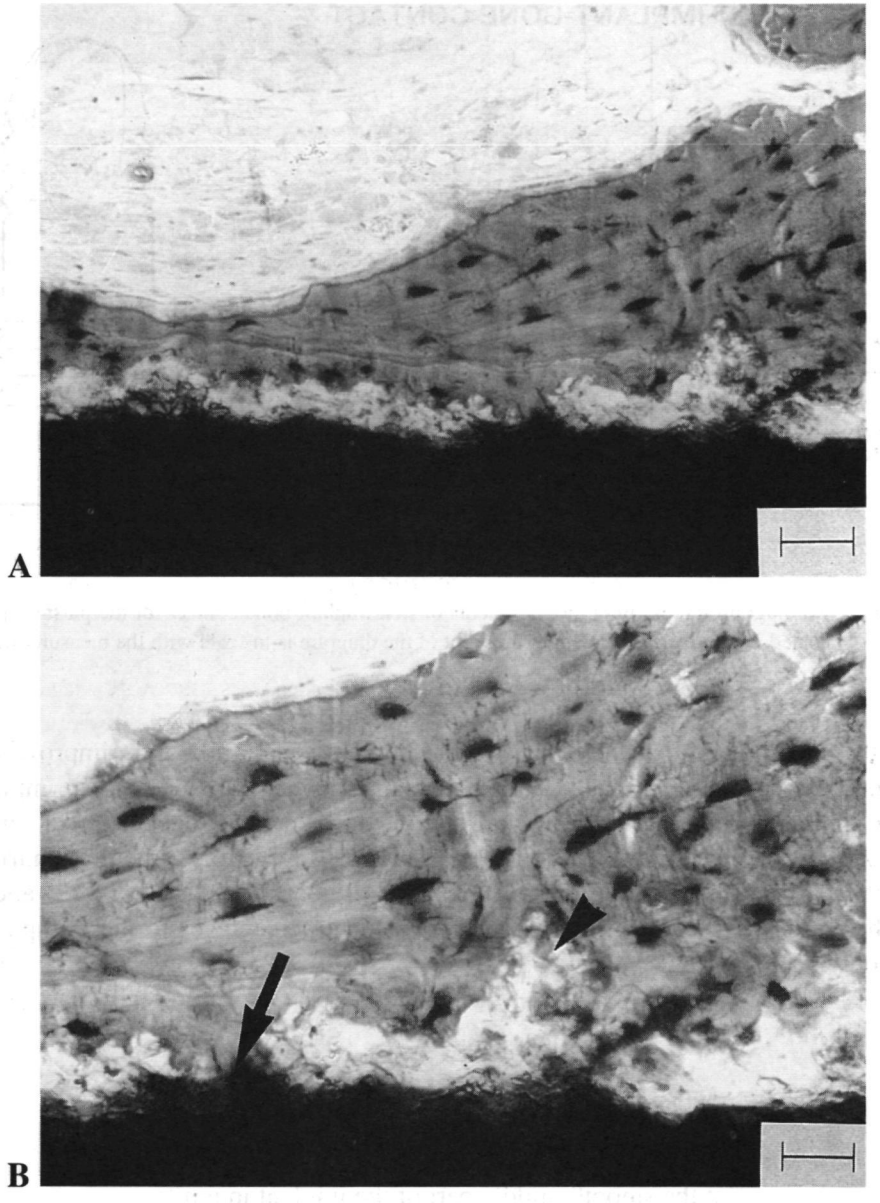

Figure 6 FA/HA coated implant after 6 months of implantation. Degradation of the coating is not uniform. (Original magnification x 63. Bar = 48 µm.) Detail: both extremes are shown, complete absence of coating (arrow) or entire thickness (arrow head) maintained. (Original magnification x 100. Bar = 30 µm.)

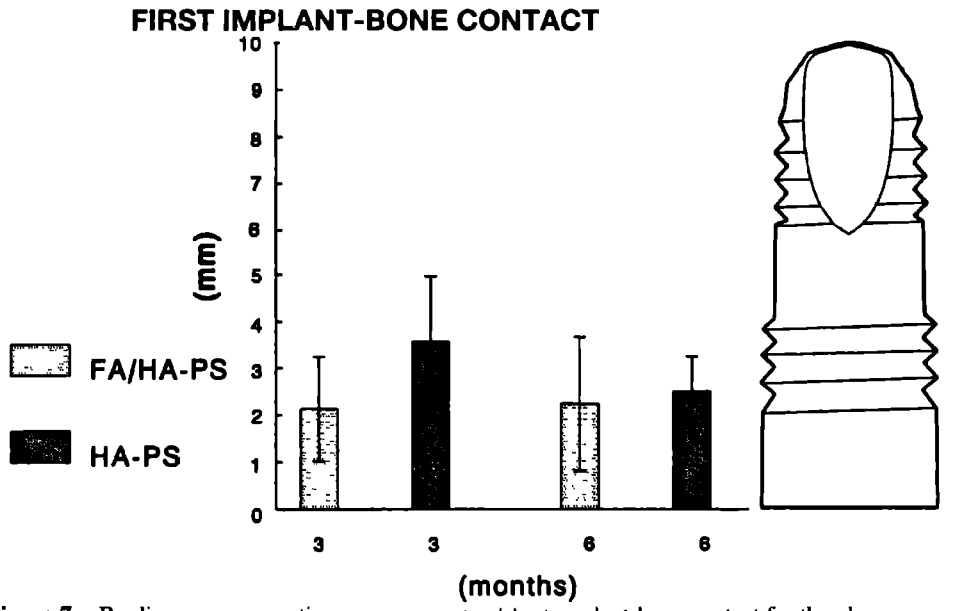

Figure 7 Bardiagram representing measurements of first implant-bone contact for the plasma spray coated implants. The implant at the right of the diagram is in scale with the measurements.

al. (1991a,b) showed that the design of the implant can significantly improve the implant performance. Optimal results are obtained with a tapered conical implant shape similar as our implant. Support for the influence of the design factor is provided by a recent study of Caulier *et al.* (1996). In this experiment cylindrical HA plasma-spray coated implants were placed in goat maxillae also for 3 and 6 months periods. However, the average bone contact percentages to these implants were only 35%.

TABLE 3

degradation of plasma spray coatings.
mean values represent the thickness of the plasma spray coating
at the smooth middle part of the implant in μm

	3 months		6 months	
	mean	std	mean	std
FA/HA-PS	25.8	7.6	20.0	11.0
HA-PS	23.7	13.2	17.3	10.7

Finally, the histological evaluation confirmed earlier reports about the degradation behaviour of plasma-sprayed Ca-P coatings. Both coatings showed a similar degradation pattern. After 6 months of implantation significant amounts of coating were left at the implant surface. Since some concern is raised about the fatigue strength properties of maintained plasma-sprayed Ca-P coatings in load bearing applications (Kangasniemi, 1994), the final consequences of this observation are not understood. Long-term loaded studies have to be performed to provide this answer.

According to the above mentioned, the clinical potential of FA/HA dual coatings is not clear. The supposed advantage of these coatings is based on an improved bone healing response, as evoked by dissolution of the amorphous HA component followed by the biostable FA part of the coating. Nevertheless, in our study, no difference was observed in coating reduction or bone contact between HA-PS and FA/HA-PS coatings after 6 months of implantation.

On basis of the findings we conclude that the bioactive properties of amorphous Ca-P sputter coatings can be questioned. Apparently, determined by the bone conditions of the implant location, crystalline sputtered coatings are required to stimulate carbonate apatite deposition *in vivo*. Further, although extrapolation of animal studies to the human situation is always complicated, we suggest that it is not necessary to wait for 6 months before loading a maxillary implant, when this implant is provided with an amorphous plasma-sprayed HA coating.

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CHAPTER 5

A mechanical and histological evaluation of Ca-P plasma spray and magnetron sputter coated implants in trabecular bone of the goat

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5.1 INTRODUCTION

Currently, the plasma-spray technique is the most frequently used method to produce Ca-P coatings. Faster and greater bone adaption, improved implant fixation and faster bone healing are described advantages of plasma spray Ca-P coatings (Cook, 1988a; Klein, 1991a; Gottlander, 1992; Dhert, 1993). Besides these benefits, concerns have been raised regarding the viable use and long-term stability of plasma-spray Ca-P coatings. Different mechanisms of coating loss, unknown long-term consequences and confusion about the optimum substrate texture advanced the investigation of other coating techniques. Since 1991, in our laboratory radiofrequency (RF) magnetron sputtering has been extensively investigated to produce thin, adherent and uniform Ca-P coatings for oral implants (Jansen, 1993; Wolke, 1994; van Dijk, 1995,1996; Hulshoff, 1995). Previous gritblasting procedures for mechanical retention, as required for plasma-spray coatings, are not needed, and physicochemically better-defined Ca-P coatings can be produced. In a previous *in vivo* experiment using rabbits (Hulshoff, 1996a) we investigated the bone behaviour to these plasma-spray and RF magnetron-sputter Ca-P coatings. Implants were inserted in the trabecular femoral bone. After 6 and 9 weeks of implantation measurements of bone contact did not reveal significant differences between the plasma-spray and RF magnetron sputter coatings. On the other hand, results of an experimental study using goats (Hulshoff, 1996b), showed better results for plasma-spray Ca-P coated implants. In this study implants were positioned in the low-density bone of the goat maxilla. In addition, there was a difference in surface roughness between the plasma-spray and magnetron sputter-coated implants. Since both of these conditions could possibly have influenced the final bone response, the aim of this study was to evaluate the bone response to Ca-P plasma-spray and RF magnetron sputter coated implants with comparable roughness.

5.2 MATERIALS AND METHODS

5.2.1 Implant materials and coating characteristics

Thirty-six tapered, conical, screw shaped dental implants (Biocomp® Industries, The Netherlands) were used. All implants measured 10 mm in length and had a diameter of 3.9 mm (Figure 1). These screws consisted of two threaded parts and a smooth middle part. They were grit blasted to a roughness of R_a 4-5 µm. Using the plasma spray method, 18 implants were provided with a 50 µm thick, bilayered Ca-P coating, consisting of fluorapatite and hydroxyapatite (FA/HA-PS) (R_a = 8-9 µm). The other 18 implants first received a 50 µm thick plasma spray

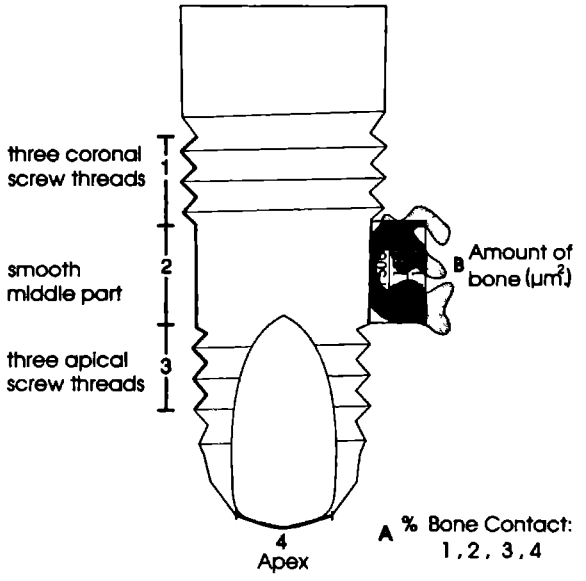

Figure 1 Schematic drawing of the tapered conical screw type implant. A and B are areas of interest for histomorphometrical evaluation.

titanium coating. Subsequently, they were covered with an additional thin film of RF magnetron-sputtered calcium phosphate (TPS/Ca-P-a) with a thickness of about 2.0 μm. The sputtering process was performed using standard conditions (background pressure: $P<8.10^{-6}$mbar, argon flow: $P=5.2.10^{-3}$mbar, power level: P=800W). The final roughness of these implants was R_a = 6-7 μm. The diameter of all implants was 4.0 mm, measured at the neck of the implant.

The chemical composition of the coatings was confirmed by X-ray diffraction spectrometry (XRD), Fourier Transmission Infrared spectroscopy (FTIR) and Rutherford Backscattering Spectrometry (RBS) measurements. XRD of the magnetron sputtered Ca-P film that covered the (TPS/Ca-P-a) coating showed an amorphous structure (Figure 2). FTIR spectra of the sputter Ca-P coating revealed PO-bonds and a large H_2O region. Ca/P ratio was 2.2. XRD (Figure 2) of the plasma sprayed FA part of the dual coating revealed >98% crystallinity, and the HA part of this coating was 60% crystalline. FTIR showed partial dehydroxylation for the HA part of the FA/HA-PS coatings. Ca/P ratio was 1.67.

Scanning Electron Microscopy (SEM) was used to record the surface appearance of the coatings.

After the coating procedure, all implants were sonicated in ethanol 100% for 10 minutes to remove any loose particles. Finally, they were sterilized in an autoclave.

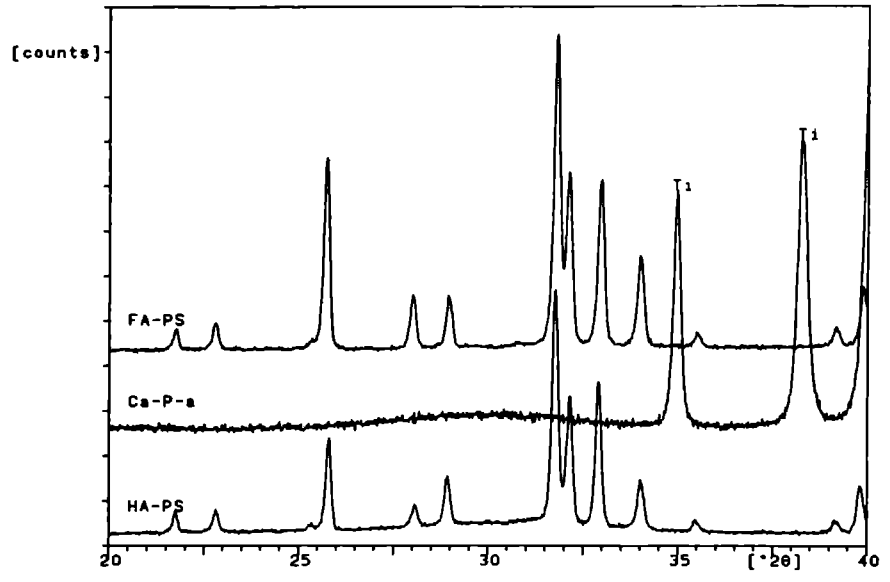

Figure 2 XRD patterns of the different Ca-P coatings with the 2 Θ in degrees on the x-axis, and the relative counts on the y-axis.

5.2.2 Experimental design and surgical procedure

Nine healthy, mature (2-4 years of age), female Saanen goats, weighing about 60 kg, were used. Blood samples of the goats were taken to ensure that the animals are CAE/CL arthritis-free. The animals were housed in a stable. The implants were inserted into the trabecular bone of the femoral condyle. The operation was performed under general anaesthesia. The anaesthesia was induced by an intravenous injection of pentobarbital and maintained by ethrane 2-3% through a constant volume ventilator, administered through an endo-tracheal tube. The goats were connected to a heart monitor.

To reduce the risk of peri-operative infection, the goats were treated according to the following doses of antibiotics:

* before the operation: Albipen 15%, 3 mL/50 kg s.c.
* 1 day after the operation: Albipen LA, 7.5 mL/50 kg s.c.
* 3 days after the operation: Albipen LA, 7.5 mL/50 kg s.c.

For the insertion of the implants, the animal was immobilized on its back and the hindlimbs were shaved, washed and disinfected with povidine-iodine. A longitudinal incision was made on the medial and lateral surface of the left and right femur. After exposure of the femoral condyle a 1.6 mm pilot hole was drilled. The hole was gradually widened with different drills to the final diameter (4.0 mm) of the implant. The bone preparation was performed with a very gentle surgical

technique, using low rotational drill speeds (max. 450 rpm) and continuous internal and external cooling. Following insertion of the implant the soft tissues were closed in separate layers using resorbable Vicryl 3-0 sutures.

A total of 36 implants were placed: 18 coated FA/HA-PS and 18 TPS/Ca-P-a implants. Each goat received 4 implants, medially and laterally positioned in the condyles of the right and left femur. The implants were inserted following a randomization scheme.

At the predetermined endpoint of the experiment, at twelve weeks, the animals were killed by an overdose Nembutal. Subsequently, both femurs were excised. Of each goat one femur was used for histological examinations, and the other femur for mechanical evaluation; to perform torque measurements and subsequent scanning electron microscopical (SEM) evaluation. In addition, of three randomly choosen goats, regional lymphnodes of both legs were excised, to inspect for the presence of titanium particles.

5.2.3 Mechanical testing

To determine the bone-bonding strength of the implants, mechanical tests were performed. After sacrificing the goat, one femur was stored and transported on ice at a temperature of approximately 4 °C. Using a diamond saw, the femur was sectioned into two pieces, each with one implant. Of these pieces containing the implants, the bone overgrowth was removed and the coverscrew of the implants was exposed and removed. This part of tissue was embedded in a mould with gypsum. A specially designed device was used to fix the specimens in the tensile bench (Figure 3). This device always enables to apply a perpendicular force (at a constant displacement speed of 0.5 mm/min) on a lever fixed in the centre of the implant. This force was applied at 1.5 cm distance of the diameter of the implants. The force at the point of implant failure was registered and the test was stopped immedialtely afterwards, to not completely fracture the interface. All samples were tested freshly.

5.2.4 Scanning electron microscopy

Following the torque measurement, specimens were fixed and dehydrated by a graded series of ethanol and embedded in methylmethacrylate. After polymerization the samples were hemi-sectioned perpendicularly on the longitudinal axis of the implants. After polishing, they were examined with a SEM in the backscatter mode to determine the fracture plane of the mechanically tested implants.

5.2.5 Histological procedures

For histological procedures 10% buffered formalin solution was used for fixation of the tissue. After fixation, each femur was sectioned into two pieces,

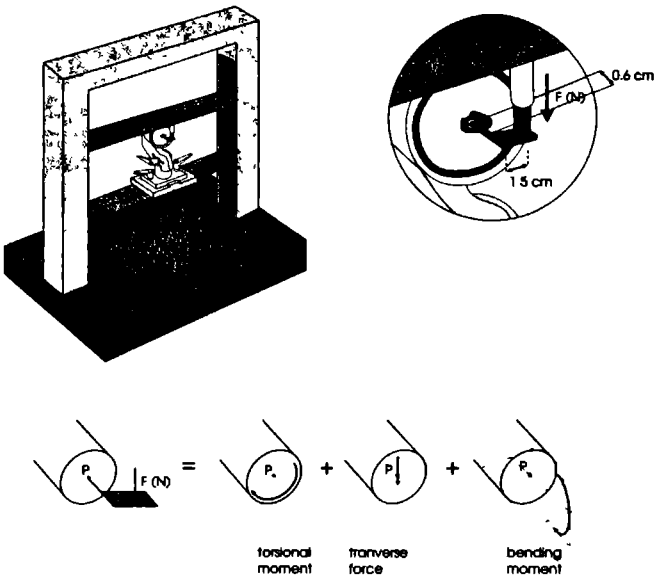

Figure 3 Schematic drawing of the torque test and mounting in the tensile testing bench. The applied force resulted in a transverse force, a bending moment, and a torsional moment, relative to point P. The bending moment and the transverse force have only minor contribution to the failure of the implant.

each with one implant. These tissue blocks were dehydrated in series of ethanol and embedded in methylmethacrylate. After polymerization nondecalcified thin (10 µm) sections were prepared in a transversal dissection perpendicular on the axis of the implant, using a modified sawing microtome technique (Klein, 1994). The sections were stained with methylene blue and basic fuchsin and the interface was investigated with a light microscope.

Excised and fixed lymphnodes were dehydrated and embedded in Paraplast. Subsequently sections were cut with a microtome (± 5 µm thick), and stained with a heamatoxylin eosin (HE) staining according to Mayer, and investigated for titanium particles, using a light microscope.

5.2.6 Histomorphometrical evaluation

Image analysis techniques (TCL-image; Technical Command Language, developed by TNO, Delft, The Netherlands) were used for histomorphometrical evaluation. The following quantitative parameters were assesed:

A. *percentage of bone contact at the interface*

Measurements were performed: 1. along three coronal screw threads, 2. at the

smooth middle part, and 3. along three apical screw threads of the Biocomp® implant and 4. along the apex (Figure 1). The amount of bone contact was defined as the percentage of implant length at which there is direct bone-to-implant contact without intervening soft tissue layers.

B. *amount of bone around the smooth middle part*

The bone amount in rectangular regions in proximity of the smooth middle part of the implant was determined. Three regions of interest (roi); roi 500, roi 1000 and roi 1500, were marked (Figure 1). All measured surfaces were rectangular with a width of 0.5 mm and a length of 2.0 mm, and a total area of 1 mm^2 (= $1.10^6 \mu m^2$). Area roi 500 was defined as a rectangular surface in direct contact with the smooth middle part of the implant. Area roi 1000 was determined at 500-1000 µm distance from the implant surface, and area roi 1500 at 1000-1500 µm distance. The amount of bone was quantified in $\mu m^2.10^3$.

All quantitative measurements were performed for 2 different sections per implant, at both sides of the implant. Results are presented based on the average of these measurements.

5.3 RESULTS

5.3.1 Mechanical testing

Table 1 shows the failure load ± standard deviation for both implants. Although, the TPS/Ca-P-a coated implants appear to show a higher failure load, statistical analysis (Student t-test) demonstrated that this difference was not significant (P>0.5).

5.3.2 SEM

Both types of implants showed good interfacial bone contact. New bone formation was observed without intervening soft tissue layers. Signs of degradation of the FA/HA-PS coating were visible. After 3 months of implantation on the TPS/Ca-P-a implants, no remnants of the amorphous sputter coating were shown by SEM. Apparently the Ca-P-a coating was disappeared. The fracture plane for these implants was situated at the bone-Ti-coating interface (Figure 4). Only

TABLE 1

	n	Failure load (N)
FA/HA-PS	9	7.71 (±3.44)
TPS/Ca-P-a	9	10.64(±4.82)

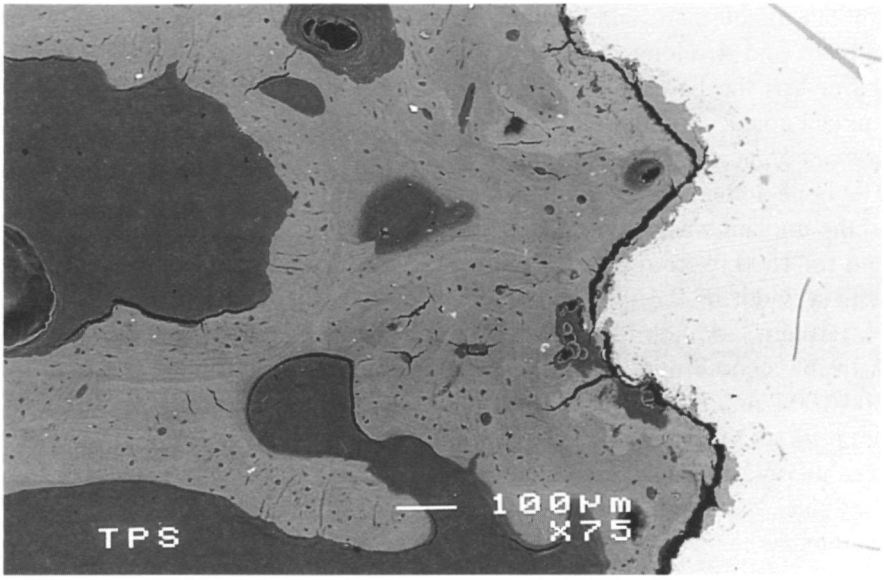

Figure 4 SEM micrograph taken in backscattermode of an implant with a TPS/Ca-P-a coating after the mechanical torque test. The fracture line is situated at the bone-coating interface. Occasionally parts of bone are included inside the fracture. Bar represents 100 µm.

occasionally a small Ti plasma-spray particle was included in this fracture. For the FA/HA-PS coating the line of fracture was less predictable. In general, at the smooth middle part of the screw implant, the fracture was situated at the implant-coating interface. At the threaded parts the fracture site was observed to occur at 3 different locations: at the implant-coating interface, inside the coating, and at the bone-coating interface (Figure 5). For both coatings, at some locations parts of bone were included within the fracture.

5.3.3 Histological evaluation

Light microscopical analysis demonstrated uneventful healing of all implants without signs of inflammation. Around both the FA/HA-PS and TPS/Ca-P-a coated implants an intimate bone-implant contact was formed (Figure 6A and 6B). Remodeling lacunae with osteoblasts were clearly visible. For the FA/HA-PS coated implants the bone around the implants appeared to have a trabecular structure (Figure 7). Around the TPS/Ca-P-a coated implants a denser type of bone was observed in close proximity of the interface (Figure 8). Around all implants the original drill margins were still recognized.

Qualitatively, FA/HA-PS coatings showed signs of degradation (Figure 7). At some sites the complete coating had disappeared, while at other spots it was present

Figure 5 Backscatter SEM picture of a FA/HA-PS coated implant. The fracture runs mainly at the implant-coating interface. Bar is 1.00 mm.

at full thickness. Frequently, loose coating particles were observed in the tissue surrounding the implants. These particles could not be associated with cellular activity (Figure 9). Further investigation revealed that these particles were an artefact caused by the sawing procedure.

Sections of the lymphe nodes did not show any sign of titanium particles.

5.3.4 Histomorphometrical evaluation

Table 2 shows the results of the measured percentages of bone-implant contact. Statistical testing (Student *t*-test) revealed a significant ($P<0.05$) higher percentage of bone contact for FA/HA-PS coated implants, in regions 2, 3 and 4. For contact area 1, the coronal screw threads, no significant difference could be measured ($P>0.05$).

Table 3 shows the results of the amount of bone in different regions of interest. Statistical testing (analysis of variance (ANOVA) and Tukey Multiple Comparison) revealed for all regions a significant ($P < 0.05$) greater amount of bone around TPS/Ca-P-a coated implants. For both implants significant differences excisted between the regions: roi 500 > roi 1000 > roi 1500.

TABLE 2

Percentages of measured bone-implant contact.
For each type of coating **n=9**
Two histological sections per implant were measured.

	FA/HA-PS	**std**	**TPS/Ca-P-a**	**std**
% contact area 1	81.31	±16.74	67.28	±22.87
% contact area 2	70.45	±11.47	34.76	±16.95
% contact area 3	62.62	±8.76	40.26	±21.81
% contact area 4	60.94	±25.98	31.12	±17.01

TABLE 3

Measured amount of bone in $\mu m^2 . 10^3$
For each type of coating and roi n=9
Two histological sections per implant were measured.

	roi 500	**std**	**roi 1000**	**std**	**roi 1500**	**std**
FA/HA-PS	518.1	±118.8	438.2	±86.1	395.4	±92.8
TPS/Ca-P-a	685.8	±45.87	532.6	±128.1	466.8	±117.7

5.4 DISCUSSION and CONCLUSIONS

The aim of this study was to investigate the bone response to Ca-P plasma-spray and RF magnetron-sputter coated implants with comparable roughness. Tapered conical screw-designed implants were installed in the trabecular bone of the femurs of 9 goats. These implants were evaluated histologically and mechanically after 3 months of implantation.

The most frequently used method to measure bone bonding strength is a push out test (Dhert, 1991; Cook, 1988b; Geesink, 1987; Klein, 1991b; Thomas, 1985). For this test, the implant is pushed out from the enclosing bone using a tensile testing bench. From the peak force, that results in movement of the implant, the interfacial shear or bonding strength can be estimated. Obviously, the push out model is only suited for cylindrical non-threaded implants. Consequently, in our study we performed a mechanical test, at which implant loosening was due to torsion. Various studies describe the use of a Jonichi® torque gauge instrument to measure the moment of torsion of threaded implants (Morberg, 1991; Wennerberg, 1995, 1996a,b; Carlsson, 1988; Gotfredsen, 1992). This value can directly be read on the instrument. Mostly this equipment is managed manually. A disadvantage of this method is that by manual operation of the Jonichi® torque gauge instrument,

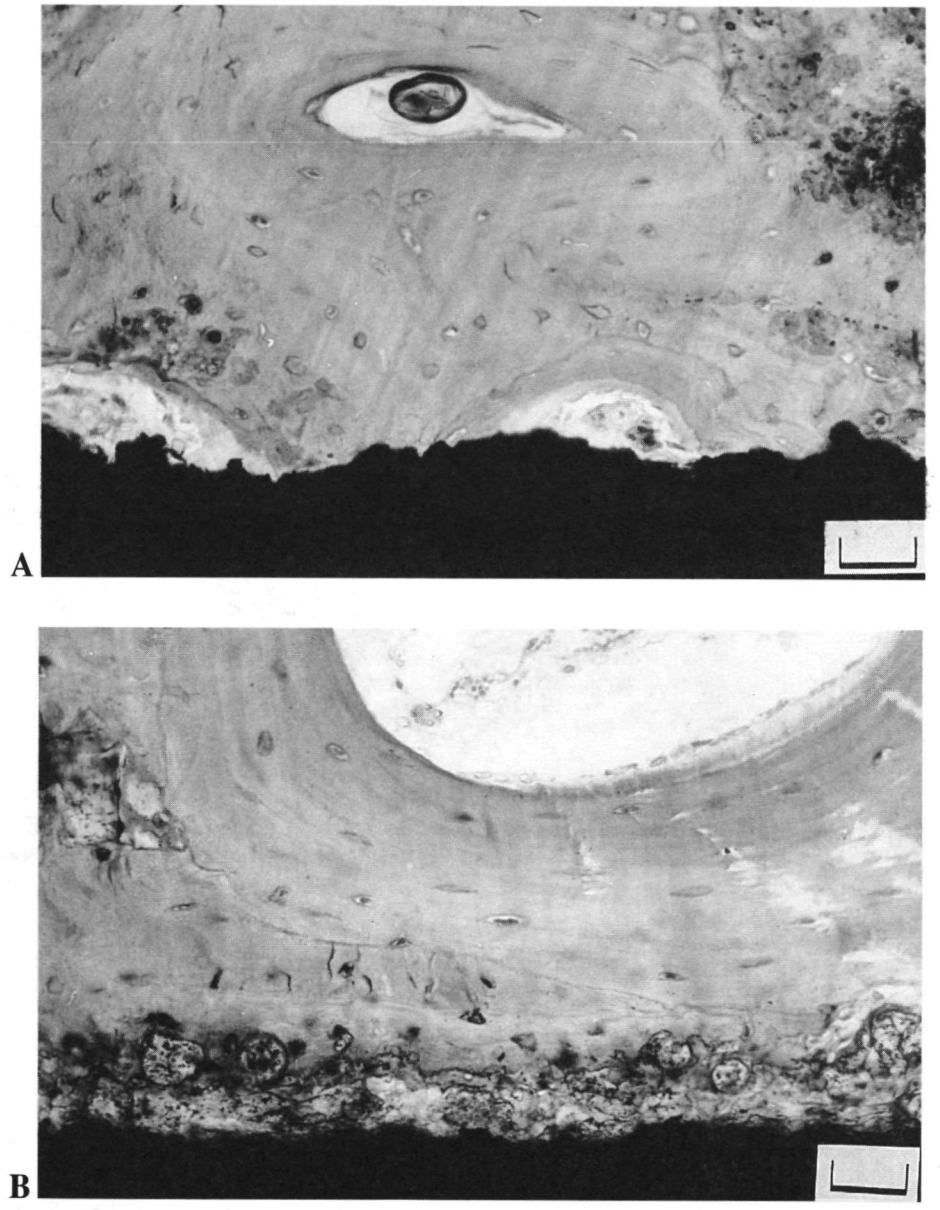

Figure 6 Light micrograph of a TPS/Ca-P-a (A) and a Fa/HA-PS (B)-coated implant; close bone apposition is shown for both types of coatings. Bar = 48 μm.

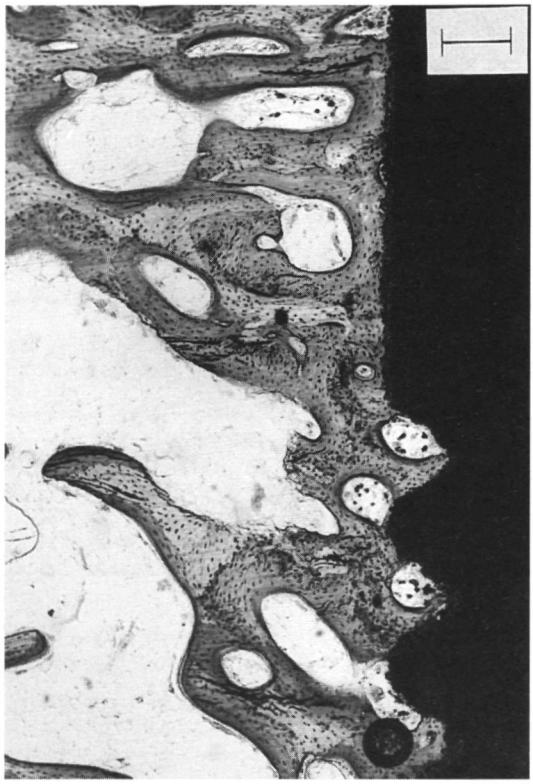

Figure 7 Histological section of an FA/HA-PS-coated implant after 3 months of implantation. In the screw threads the coating has partially disappeared. The trabecular structure of the bone around the implant can clearly be recognized Bar = 300 μm.

additional shear stresses are induced. These will never be exactly the same for each measurement. Further, it is very difficult to obtain and maintain a correct perpendicular alignment of this measuring device on the implant during unscrewing. Therefore, we developed a special device, which makes it possible to apply and maintain an exact reproducible perpendicular force on the threaded implants. This force was applied on a lever at 1.5 cm distance from the centre line and 0.6 cm in front of the implant. The load results mostly in torsion at the implant, which is now well controlled by the testing apparatus. In addition, small shear and bending stresses are induced (Figure 3), but these are also well controlled and identical for all specimens. Another advantage is that it is not necessary to unscrew the implant completely, since the force can be stopped immediately after the bone bonding is partially fractured. This allows investigation of the processes at failure by SEM. Finally, it has to be emphasized, that caution must be taken to compare different

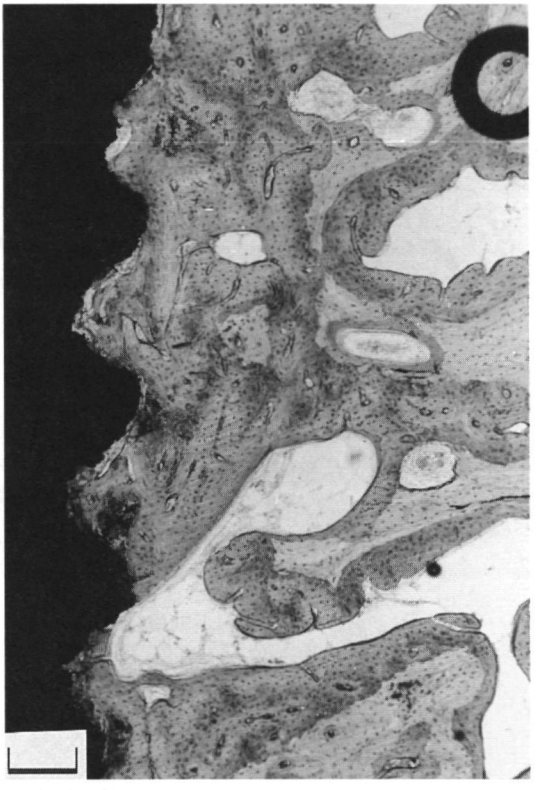

Figure 8 Light micrograph showing a denser type of bone in close proximity to a TPS/Ca-P-a coated implant, 3 months after implantation. Bar = 300 μm.

studies. Measured values are relative and only conclusive for the described type and design of implant, in the described type of bone (Dhert, 1992; Berzins, 1996). For correct interpretation of different studies a biomechanical evaluation by a finite element analysis of the different tests will be necessary (Berzins, 1996). However, this exceeds the aim of this study.

Using our test design, the mechanical evaluation showed that a biomechanical interlocking and perhaps even a chemical bonding developed between the TPS/Ca-P-a implants and surrounding bone, as depicted by the fracture in the interface bone-coating. In contrast, the resistance to torsional force appeared to be inadequate for plasma-sprayed FA/HA-PS coatings, since fracture in the interface coating-implant occured. Such an inadequate attachment between Ca-P plasma-sprayed coatings and titanium has already been described earlier in pull-off tests (Kangasniemi, 1994) and *in vivo* animal experiments (Clemens, 1995). The supposed advantage of dual FA/HA-PS coatings, based on an improved bone healing response by fast dissolution

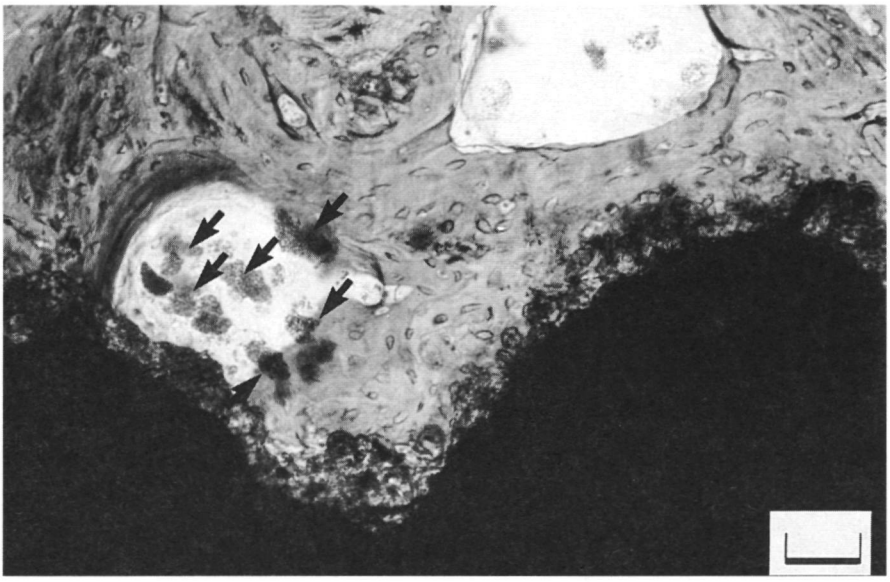

Figure 9 Microscopical picture showing FA/HA-PS particles (arrows) which are detached from the implant surface. No cellular activity is visible around the coating particles. Further examination revealed that this is an artefact caused by the used histological sectioning technique. Bar = 75 mm.

of the amorphous HA component and subsequent maintenance of the biostable FA component appears to be overruled by the unfavorable adhesion properties of these coatings to the implant. Therefore, we conclude that the rationale for the use of such dual coatings in loaded situations can be questioned.

After 3 months of implantation the Ca-P-a sputter coating was not detected by SEM. This was confirmed by recent *in vitro* and *in vivo* dissolution experiments (Wolke, 1996). In these studies we demonstrated that complete dissolution of 2-4 µm thick amorphous Ca-P sputter coatings occurs. This in contrast to crystalline coatings. Therefore, we think that the bioactive behaviour of Ca-P sputter coatings is, among others, determined by their crystallinity. Corten (1995) described the phenomenon of a lower percentage of bone apposition, in combination with a higher bone density for uncoated Ti implants, compared to various plasma-spray Ca-P coated. Our measurements of the amount of bone also revealed significantly more bone around TPS/Ca-P-a coated implants in combination with less bone contact, then for FA/HA-PS coated implants. The possibility of a persisting bone reaction influenced by the implant material can not be excluded. Consequently, a less than ideal bony integration will result in a qualitatively less stress transferring system. This implies that the implant will act as a constant mechanical stimulus (Williams, 1990). As a

result of this persisting trauma the bone turnover around the implant will be increased, as characterized by an increased bone mass. This observation again pleads in favor for the use of well characterized, stable Ca-P coatings, especially when degradation or mechanical properties of such coatings can be controlled or improved.

Finally, Weingart et al (1994) described the transportation of fine titanium particles by phagocytes to regional lymphe nodes. These particles were supposed to be loosened during insertion of titanium plasma-spray coated implants. Despite this observation, our study did not reveal the presence of titanium particles in the regional lymphe nodes. Although an explanation for this descrepancy in observations is difficult to give, we suppose that differences in the manufacturing process or cleaning procedures can be a reason for the presence of loose particles. Nevertheless, concerns about loose and transported particles of titanium plasma-spray coatings appears to be justified and should always be thoroughly investigated.

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CHAPTER 6

Initial interfacial healing events around calciumphosphate (Ca-P) coated oral implants

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6.1 INTRODUCTION

It has generally been accepted that calciumphosphate (Ca-P) coated implants induce a faster bone adaptation and improved bone healing (Dhert 1993; Cook 1988; Gottlander 1992; Caulier 1995, 1996; Hulshoff 1996a). Currently, the plasma-spray technique is the most widely used technique for the deposition of Ca-P ceramic coatings (Wolke 1992; Dalton 1995). However, recently some concerns have been raised about the safety and clinical prognosis of plasma-spray Ca-P coated oral implants (Wheeler 1996). Failures have been reported, which mainly have to do with the degradation and fatigue behavior of these coatings (Frayssinet 1995; Bloebaum 1993, 1994). This can hamper the further use of Ca-P implants. Consequently, experiments are started to improve the biodegradation of plasma-sprayed Ca-P ceramic (Klein, 1993). For example, to increase the stability, additional heat treatment procedures and various Ca-P powder compositions are used. The rationale behind these modifications is, that degradation is related to the structural properties (amorphous vs. crystalline) of the coating. On basis of the different studies, de Groot (1994) concluded that high coating crystallinity does not improve compatibility, but rather reduces the bioactivity compared with amorphous coating. Therefore, he suggested that perhaps the most optimal coating should consist of: (a) an amorphous outer layer for the bone healing, and (b) a crystalline inner layer to reduce the degradation.

Besides plasma-spraying, also other techniques can be used to produce Ca-P ceramic coatings. For this purpose, since 1991, in our laboratory efforts have been made to the development of the so-called radio frequency (RF) magnetron sputter method. The advantages of this process are that the deposited films are very thin (100 nm-4 μm), well-defined in structure and composition, and strongly bonded to the underlying metal substrate. Previous cell culture and animal experiments (Hulshoff 1995, 1996b) already showed the biological feasibility of this type of coatings.

Finally, it has to be noticed that, despite the frequently described favorable bone reaction, the reason for the beneficial effect of Ca-P ceramic is still not completely understood. It is only suggested, that events in the cellular and tissue response during the initial healing phase are responsible (LeGeros, 1993).

Therefore, the aim of this investigation was to compare the early interfacial response to two alternative Ca-P coated implants with a non-coated control.

6.2 MATERIALS AND METHODS

6.2.1 Implant materials and coating characteristics

Fifty-four tapered, conical, screw shaped dental implants (Biocomp® Industries) were used. All implants measured 10.0 mm in length. Thirty-six

implants had a diameter of 4.0 mm and 18 implants had a diameter of 3.9 mm. The implants with a 3.9 mm diameter were grit-blasted to a roughness of R_a = 4-5 µm. They were cleaned ultrasonically in propanol, and dried at 100 °C. Subsequently, these implants were provided with an approximately 60 µm Ca-P coating using a plasma-spray process. An experimental bilayered Ca-P coating was deposited. The inner 30 µm consisted of crystallline fluorapatite (FA) and the outer 30 µm of amorphous hydroxylapatite (HA). The final surface roughness of these FA/HA-PS coatings was R_a = 9.59 µm. Further, of the implants with a diameter of 4.0 mm, 18 were left uncoated (cpTi) and 18 were provided with an amorphous Ca-P (Ca-P-a) coating using a RF magnetron sputter process (van Dijk 1995a, 1995b; Wolke 1994). These implants were not grit-blasted, but were argon-etched before sputter coating. The produced coatings had a thickness of 2-4 µm. Final roughness for cpTi and for Ca-P-a implants was respectively: R_a = 0.44 µm and R_a = 0.40 µm. The final diameter of all implants was 4.0 mm (± 0.1 mm).

Before implantation, all implants were sterilized by autoclave.

6.2.2 Experimental design and surgical procedure

Eighteen healthy, mature (2-4 years of age), female Saanen goats, weighing about 60 kg were used. Prior to the experiment, blood samples of the goats were taken to ensure that the animals were CAE/CL arthritis-free. All animals were housed in a stable.

The implants were inserted into the trabecular bone of the femoral condyle. The operation was performed under general anaesthesia. The anaesthesia was induced by an intravenous injection of pentobarbital and maintained by ethrane 2-3% through a constant volume ventilator, administered through an endo-tracheal tube. The goats were connected to a heart monitor. To reduce the risk of peri-operative infection, the goats were treated according to the following doses of antibiotics:

* before the operation: Albipen 15%, 3 ml/50 kg s.c.
* one day after the operation: Albipen LA, 7.5 ml/50 kg s.c.
* three days after the operation: Albipen LA, 7.5 ml/50 kg s.c.

For the insertion of the implants, the animal was immobilized on its back and the hind limbs were shaved, washed and disinfected with Betadine-iodine. A longitudinal incision was made on the medial and lateral surface of the left and right femur. After exposure of the femoral condyle, 1.6 mm pilot holes were drilled. These holes were gradually widened with different drills to the final diameter (4.0 mm) of the implant. The bone preparation was performed with a very gentle surgical technique, using low rotational drill speeds (max. 450 rpm) and continuous and external cooling. Following insertion of the implant the skin was closed using Vicryl 3-0 sutures.

A total of 54 implants was placed: 18 uncoated cpTi, 18 coated FA/HA-PS, and 18 Ca-P-a implants. Each goat received 3 implants. The implants were inserted following a balanced split plot design, with one vacant position.

The protocol did foresee to kill six goats after 3 days, six after 12 days and six after 24 days using an overdose of Nembutal®.

6.2.3 Histological procedures

Following the death of the goats, the femoral condyles were excised and fixed in a 10% buffered formalin solution. Then the remaining tissue blocks were dehydrated in a series of ethanol. Finally, they were embedded in methylmetacrylate. Using a modified diamond blade sawing microtome technique (vd Lubbe 1988, Klein 1994), non-decalcified thin sections were made. These were stained with methylene blue and basic fuchsin to be examined by a light microscope.

6.2.4 Histological evaluation

The trabecular bone response to the implants was assessed histologically. First, for all implantation periods a descriptive evaluation was performed. Second, for the 24 days implants also histomorphometrical measurements were done. Therefore a computer based image analysis system (TCL-image) was used. Microscopic images were projected on a monitor. For this purpose, a video camera was coupled to the light micrope (magnification 2.5x1.25). In this way the percentage of direct bone contact at the interface for 4 different areas of interest was determined. These areas were (Figure 1):

1. along three coronal screw threads
2. at the smooth middle part
3. along three apical screw threads
4. along the apex

All quantitative measurements were performed for 2 different sections per implant. Presented results are based on the average of these measurements.

6.3 RESULTS

During the experimental period, one of the goats of the 24-day group died because of an acute peritonitis caused by a pancreatitis. The other animals remained in good health during the various test periods. At sacrifice, no clinical signs of inflammation or adverse tissue reaction could be seen. All implants were still *in situ* at sacrifice.

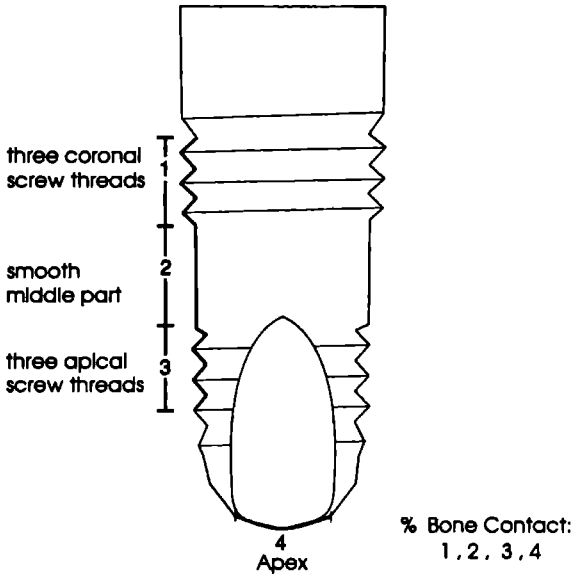


Figure 1 Schematic drawing of the tapered conical screw type implant. Percentages of bone contact were determined at 4 different areas of interest.

6.3.1 Light microscopical evaluation

After 3 days of implantation no difference in tissue response between the various implants could be observed (Figure 2). The original hole, that was drilled to place the implant, could be recognized very well. In some cases the implant did not reach the bottom of the hole. Then, the available space around the implant and between the original bone trabeculae was filled with splinters of bone as left by the drilling procedure. Further, blood coagulum, primitive bone marrow cells, and undifferentiated inflammatory cells were visible. Only few multinucleated giant cells were present.

After 12 days of implantation, around all implant materials a callus of woven bone was formed, which bridged the excisting space between bone trabeculae and implant surface. Extensive networks of active osteoblasts were seen. Bone formation was not restricted to the interface. Trabeculae of the surrounding bone, that were not traumatized by drilling, also showed an active formation of new bone. Still inflammatory cells were seen, though in a lesser extent than after 3 days of implantation. Despite this similarity in overall reaction, a clear difference existed in interfacial response between coated and non coated implants (Figure 3).

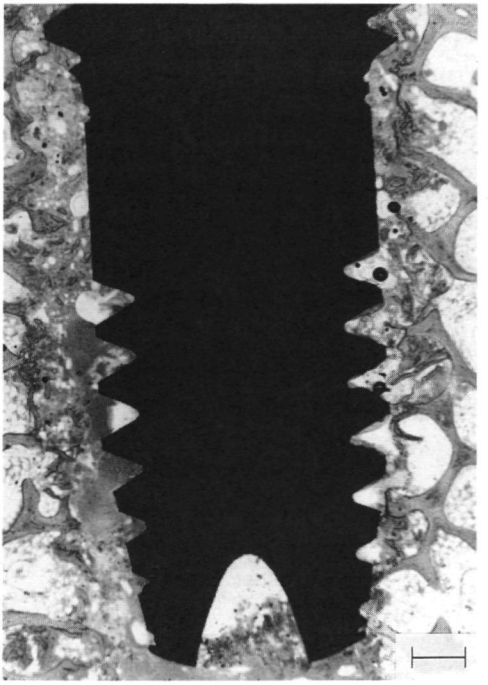
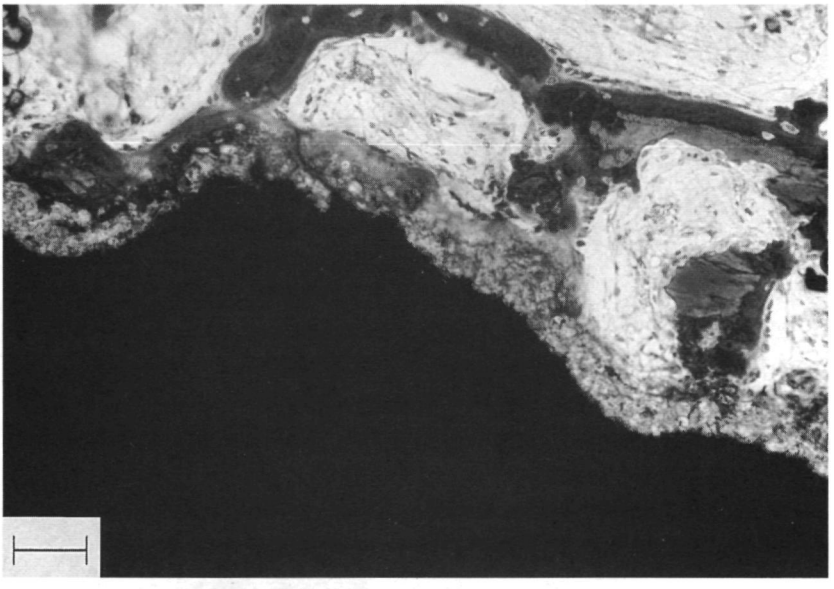


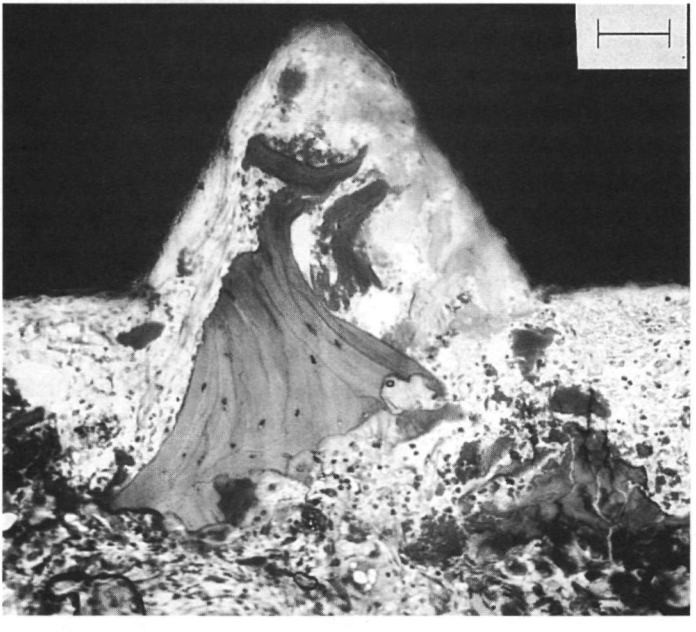
Figure 2 Light micrograph of a Ca-P-a coated implant after 3 days of implantation. The originally drilled hole is visible. The available space is filled with blood coagulum and splinters of bone as caused by drilling. (Original magnification 2.5X, bar = 400 µm.)

For both types of Ca-P coated implants osteoid appeared to be present in a greater abundance compared with non-coated implants.

After 24 days of implantation, both types of Ca-P coatings showed a lot of bone contact (Figure 4). Even if the implant apically was not in contact with the original bone, a thin layer of bone was often covering the implant. On the other hand, we noticed that the appearance of the surrounding bone for FA/HA-PS en Ca-P-a implants was different. In a region with a width of about 500 µm, the bone around the Ca-P-a implants was more dense than around FA/HA-PS implants (Figure 4). For the FA/HA-PS implants, the bone in this area was more similar to the original bone. In contrast with the coated implants, the cpTi implants did not show much bone contact. Further, around all implants still some inflammatory cells could be seen. The plasma-sprayed coating also showed signs of superficial coating degradation, as characterized by the presence of fragmented particles (Figure 5). This degradation process could not be associated with the occurrence of inflammatory cells.



A



B

Figure 3 Light micrographs of both types of coated implants after 12 days of implantation.
A. FA/HA-PS coating: newly formed bone is covering the implant.
B. Ca-P-a coating: osteoid is present in the screwthreads.
A. bar = 100 µm B. bar = 40 µm.

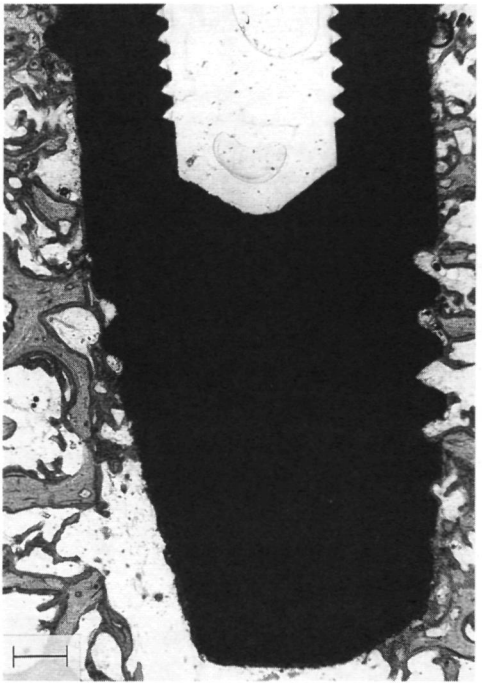


Figure 4 Light micrograph of an FA/HA-PS coated implant after 24 days of implantation. Around the FA/HA-PS coated implants the original structure of bone trabeculae around the interface is maintained. New bone formation is also formed in trabeculae that were not traumatized by drilling. (Original magnification 2.5X, bar = 400 µm.)

6.3.2 Histomorphometrical evaluation

The results of the bone contact measurement after 24 days of implantation are listed in Table 1. In all areas, the average percentage of bone contact appeared to be higher for the Ca-P coated implants than for the cpTi implants. Nevertheless, statistical analysis using an one way analysis of variance (ANOVA) and Tukey

TABLE 1

Mean Bone Apposition (%) ± Standard Deviation after 24 days of implantation (number of implants is 5)

	area 1	area 2	area 3	area 4
cpTi	41.56 (±31.46)	25.44 (±10.82)	19.57 (±15.89)	13.25 (±14.00)
Ca-P-a	68.25 (±31.56)	44.11 (±40.62)	43.58 (±36.85)	28.85 (±17.95)
FA/HA-PS	90.85 (±8.72)	84.71 (±4.98)	62.93 (±10.92)	52.84 (±26.97)

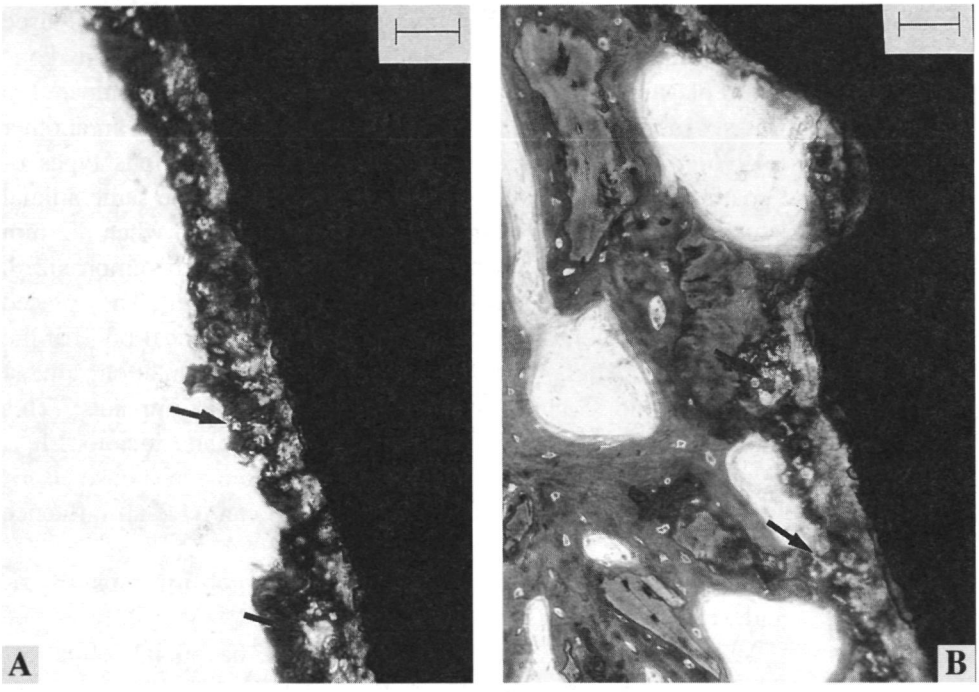


Figure 5 Light micrographs of a FA/HA-PS coated implant after 24 days of implantation. New bone has formed. The degradation associated with fragmentation can be seen. (Original magnification 20X, bar = 50 μm.)

multiple comparison procedure revealed that this difference was only significant ($p<0.05$) for the FA/HA-PS coated implants. In addition, the results show that the amount of bone contact in areas 3 and 4 was lower than in areas 1 and 2.

6.4 DISCUSSION AND CONCLUSIONS

In this study the influence of different Ca-P coatings on initial bone healing of screw type oral implants was investigated. Therefore Ca-P coated implants and non-coated cpTi implants were inserted in the trabecular bone of the femur of goats for implantation periods of 3, 12 and 24 days. It was found that Ca-P coated implants favor the early bone response. Already after 12 days of implantation the bone forming process had proceeded faster for the coated implants. This could not completely be confirmed in the bone contact measurements. Due to a wide variation in bone contact percentage, statitistical significance could only be demonstrated for the plasma-spray coated implants. Nevertheless, our findings do

corroborate with the experiments of other investigators, although they all used longer implantation periods. For example, Gottlander (1992) found more bone direct bone contact to plasma-sprayed hydroxyapatite-coated implants compared to non-coated controls six months after placement in the femur of rabbits. In another study, Clemens (1996) observed increased bone apposition to various types of calciumphospate coatings within six weeks of implantation using the same animal model as in our study. This improved bone response even occurred when a 2 mm gap was created around the implants. On the other hand, it has to be emphasized, that our results do not agree with a recent study of Dhert et al. (1996). They placed plasma-spray Ca-P coated and non-coated Ti implants into the cortical bone of the tibial metaphysis of rabbits. After 3, 7, 18 and 28 days of implantation, almost similar percentages of bone contact were measured for all implants. This discrepancy with our observations can be due to the used implant location. It is known, that trabecular and cortical bone have a different healing response (Burr, 1993). Also, the implant location (condylus vs. metaphysis) can have an influence (Dhert, 1991).

Considering the Ca-P sputter coatings, the present results confirm most of our earlier *in vitro* and *in vivo* studies (Hulshoff 1995, 1996a, 1996c). Only in one study (Hulshoff 1996c), we failed to demonstrate the beneficial effcet of amorphous Ca-P sputter coatings. In this study, sputter coated implants were inserted in the maxillary trabecular bone of goats. This bone is of very low density. As we suggested, this negative result was probably due to the difference in trabecular bone quality between the goat maxilla and femur. As a result, the amorphous coating dissolves too fast. Unfortunately, this hypothesis cannot be confirmed with quantitative data about the *in vivo* dissolution behavior of Ca-P sputter coatings in various bone locations. The films are too thin for the currently available analytical techniques.

Despite the great difference in surface roughness between both types of Ca-P coating, the sputtered implants showed a high amount of bone contact. Several studies demonstrated already, that implant surface topography effects the bone biocompatibility (Buser 1991; Wennerberg 1995a, 1995b, 1996). In a recent study, Gotfredsen et al (1995) even claimed that a rough surface is a prerequisite for the successful implantation in bone of low quality or quantity. Consequently, it can be supposed that roughening of the implant surface together with a thin Ca-P sputter coating will increase the bone apposition to the same level as for rough plasma-sprayed coatings. More research is necessary to confirm this theory.

Notwithstanding the better response to the Ca-P coated implants, we have to mention, that this difference in surface roughness can also be responsible for the very disadvantageous bone response to the cpTi implants. This suggestion is supported by the findings of Courtney (1995) and Gomi (1993). They demonstrated

that the amount and distribution of mineralized bone matrix is influenced by the substratum surface roughness.

The difference in bone contact between areas 1 and 2 and areas 3 and 4 is probably due to the surgical pocedure. In the histological evaluation we observed that the implants were not always in contact with the bottom of the drilled hole. The apical part of the implant has a conical shape. Incomplete positioning resulted in a small apical gap between implant and bone. Evidently, this reduced fit of the implant has to be responsible for the lower percentage of bone apposition.

Finally, some critical remarks have to be made about the degradation of the used bilayered FA/HA-PS coating. The amorphous outer layer was designed to improve the bone reaction. The purpose of the crystalline inner layer was to stay in place in order to maintain the optimal functioning of the implant. On basis of the bone contact measurements, it appears that the amorphous layer indeed fulfilled its role. On the other hand, we observed that the resorption was associated with a fragmentation process of the coating. Although, this could not be related with a cellular response, it cannot be excluded that at the long-term the released particles will lead to irritation. Further, from the point of bone bonding, the use of a non-resorbable layer is also uncertain. Especially, because there are some indications that maintenance of Ca-P coatings can endanger the final fixation of the implants due to delamination phenomena (Kangasniemi 1994).

Supported by the results, we conclude that: (1) the superior bone response to Ca-P ceramic coatings is due to an initial difference in bone cell response, and (2) RF magnetron sputtered Ca-P coatings can improve the biological capacity of oral implants. Further studies have to be performed to optimize the sputter technique. These experiments have to focus on the influence of coating composition (structure and Ca/P ratio), the clinically required coating thickness and the influence of the implant surface topography.

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CHAPTER 7

Interfacial phenomena: an *in vitro* study to the effect of calcium phosphate (Ca-P) ceramic on bone formation

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7.1 INTRODUCTION

The successful integration of medical implants into the body can be envisioned as a race against time among the processes of wound healing, fibrosis and microbial invasion. The stake in this contest is the realisation of a harmonious interface between the implanted material and the surrounding tissues as characterized by the development of natural tissue structures (Hjörting-Hansen, 1990). For example, biomaterials serving as permanent replacement of diseased or traumatized bone and teeth, require an interface that is contiguous with the surrounding bone and participates in the naturally occurring bone remodelling process. As is evident from previous research (Schwartz, 1994), surface properties of biomaterials play an important role in the final implant-tissue response. In this light, a lot of research has been performed to the influence of calcium phosphate (Ca-P) ceramics on bone interfacial processes. Despite a frequently noticed increase of bone apposition (de Bruijn, 1992, 1993), the available information about how Ca-P materials trigger bone formation is at best rudimentary. We can only speculate about the mechanisms underlying the improved sensitivity of osteogenic cells to these materials. One explanation is that Ca-P ceramic is bioactive due to its binding of serum proteins and growth factors (Bagambisa, 1990; Begley, 1995; de Bruijn 1995; Labat, 1995). It has also been suggested that Ca-P ceramics are the same minerals as found in bone. The chemical composition of Ca-P material is considered to play an important role in cellular production as well as maturation of extracellular matrix (ECM). It is assumed that Ca^{2+}-ions dissolve from the Ca-P surface, causing with the already present Ca^{2+}-ions an interfacial supersaturated condition, which results into the deposition of an afibrillar mineralized layer consisting of several Ca-P mineral phases (Li, 1993; Ong, 1995). This stimulates the bone cells to continue ECM (bonding zone) synthesis and calcification (Martin, 1995; Stanford, 1991). This ECM, as secreted by the cells, evolves by the fusion of individual calcium phosphate containing globular accretions (matrix vesicles) of about 100 nm diameter produced at the distal ends of the osteoblasts processes attaching to the underlying substratum. It is supposed that the presence of Ca-P ceramic has a direct influence on cellular production of matrix vesicles as well as on matrix formation.

To reduce the above mentioned lack of knowledge we do cell culture studies in our laboratory with osteoblast-like cells on Ca-P ceramics. For this purpose, by RF magnetron sputter coating, titanium discs are covered with different thin Ca-P films. This coating technique allows the preparation of Ca-P ceramics with different stoichiometries (hydroxyapatite, tricalciumphosphate and tetracalcium-phosphate), Ca/P ratios (from 1.67 to 2.6), crystallinity states (crystalline and amorphous) and thicknesses (from 100 nm to 10 μm), while maintaining the

surface topography of the underlying substrate surface (Wolke, 1994; van Dijk, 1995a). In a first set of experiments (Hulshoff, 1995), we demonstrated that magnetron sputtered Ca-P coating can indeed induce apatite deposition and stimulate the formation of mineralized ECM.

The aim of this study was to examine the influence of Ca/P ratio on osteoblast reaction by *in vitro* experiments. Using various analytical techniques, information was obtained about: (1) the morphology of the cells and their cell-substrate interface, and (2) the structure and composition of the deposited ECM and underlying Ca-P layer.

7.2 MATERIALS AND METHODS

7.2.1 Calcium Phosphate Coatings

Round discs (diameter 12 mm, thickness 1 mm) of commercially pure titanium (cpTi) were prepared and hand finished (320 grit). They were ultrasonically cleaned in acetone for 15 minutes, and placed in 100% boiling ethyl alcohol. Thereafter, the cpTi discs were either left untreated (cpTi) or provided with three different 2.5 µm thick Ca-P coatings. The coatings were prepared, as already described earlier (van Dijk, 1995b), at the following sputter conditions:

- 400 Watt, $P_{tot}=1.5*10^{-2}$ mbar, Ar + 0% O_2 **(CaP400)**
- 400 Watt, $P_{tot}=1.5*10^{-2}$ mbar, Ar + 1% O_2 **(CaP400O2)**
- 400 Watt, $P_{tot}=1.5*10^{-2}$ mbar, Ar + H_2O **(CaP400H2O).**

All as-sputtered coatings were characterized by X-ray diffraction (XRD), Fourier transform infrared spectrometry (FTIR), and Rutherford backscattering spectrometry (RBS) (van Dijk, 1995). Before use in the cell culture experiments, discs were autoclaved for 30 minutes at 120 °C.

7.2.2 Cell Isolation and Culture

For the biological evaluation of the test materials a rat bone marrow (RBM) cell culture technique was used as described by Maniatopoulos (1988). Briefly, bone marrow cells were obtained from femora of male Wistar rats. Epiphyses were cut off, and both diaphyses were flushed out, using α-MEM (Gibco, Life Technologies B.V., Breda, The Netherlands) supplemented with 15% foetal calf serum (FCS, heat induced at 56°C for 35 min, Gibco), 50 µg/mL freshly prepared ascorbic acid (Sigma, Chemical Co., St. Louis, MO., USA), 10 mM Na ß-glycerophosphate (Sigma), 10^{-8}M dexamethasone (Sigma) and antibiotics (gentamicin and fungizone). Per femur 15 ml of this medium was used, cells were suspended and

cultured at three 80 cm^2 tissue culture flasks (Nunc Products by Gibco). Finally, cultures were incubated in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. After 5-7 days of primary culture, cells were detached using trypsin/EDTA (0.25% w/v trypsin/ 0.02% EDTA) and the cells were suspended in the supplemented culture medium as described above.

7.2.3 Proliferation Assay

Cell suspension ($4x10^4$ cells/well) was added to the four different substrates (cpTi, CaP400, CaP400O2 and CaP400H2O), which were positioned at the bottom of 24 well-plates (Greiner, Greiner B.V., Alphen a/d Rijn, The Netherlands). The cultures were incubated at 37 °C in 5% CO_2-air atmosphere. At incubation day 1, 3, 5 and 7, each time the culture medium was refreshed. After time instances 3, 5 and 8 days the cultures were washed using ISOTON II Azide Free balanced electrolyte solution (Coulter) to remove the non-attached cells. Then the substrates were taken out of the well and placed into a counting tube. Attached cells were removed with 1.0 ml of trypsin/EDTA for 7 minutes at 37°C. ISOTON II solution was added and the cells were counted immediately using a Coulter Counter. After the counting procedure the presence of non-detached cells was cheched using a methylene blue staining method and incident light microscopy. Finally, cell proliferation rates were calculated.

Two runs of experiments were carried out. In each run all materials were present in quadruple. In addition, in each run Thermanox® cover slips (Nunc Products by Gibco) were included as reference material.

7.2.4 Tetracycline Assay

In this experiment again the four different substrates and the Thermanox® reference material were tested. A fluorescent technique, as described by Todescan and Davies (1996), was used to quantify the calcified extracellular matrix produced by the RBM cells. Where bone mineral is being formed, tetracycline antibiotics bind to hydroxyapatite, through chelating with calcium. Therefore, starting from incubation day 3 tetracycline (9 µg/ml) replaced the earlier mentioned antibiotics (Fungizone and Gentamicine) in the culture medium. After 16 days of incubation, the medium was removed, the cultures were rinsed twice in 70% ethanol, and finally dehydrated in 100% ethanol at 4 °C for 6 hours. After a further dehydration in ethanol 100%, the cultures were allowed to dry in a dark room. After drying, a Biorad MRC 1000 confocal laser scanning microscope (CLSM) was used to visualize fluorescence. This CLSM was equipped with a krypton/argon mixed gas laser (Ion Laser Technology, Salt Lake City, UT, USA), which was mounted on a

Nikon Diaphot microscope with non-cover glass (NCG) objectives (Nikon). The specimens were illuminated at an excitation wavelength of 488 nm. For each substrate, 10 randomly chosen areas were imaged. The 10 areas covered in total 7.15 mm^2.

The resulting digital images were stored on hard disc. Digital image analysis of the images was performed using an Acorn® computer provided with Technical Command Language image software (TNO, Rijswijk, NL). Mineralization was quantified by counting the excited pixels and expressed as surface area in mm^2.

Two runs of experiments were carried out. In each run, all materials were present.

7.2.5 Dissolution Assay

The possible influence of culture medium on the initial dissolution behaviour of the deposited Ca-P coatings was also investigated. Therefore, coated discs were incubated for 3 and 5 days with fully supplemented culture medium without cells. A similar refreshment scheme of the medium was used as for the proliferation assay. After incubation, the samples were analysed with SEM, RBS, and FTIR.

7.2.6 Scanning Electron Microscopy

The influence of the substrate surface composition on ECM synthesis of RBM cells was studied using scanning electron microscopy (SEM). After 8 and 16 days of incubation, the non-attached cells on the various substrates (only uncoated and coated Ti-discs) were removed by rinsing twice with 0.1 M sodium-cacodylate buffer (pH 7.4, 37 °C). Subsequently, fixation was carried out for 60 minutes at 4°C in 2% glutaraldehyde in the same buffer. After dehydration in a graded series of ethanol and drying by tetramethylsilane (Merck®, Germany), the specimens were sputter-coated with gold. These gold-coated specimens were then examined and photographed using a Jeol 6310 SEM at an accelerating voltage of 15 kV.

7.2.7 Transmission Electron Microscopy and X-ray Microanalysis

RBM cell suspension was added to the substrates, as described before, and the cultures were incubated for 16 days. After the incubation period, the non-attached cells were removed by phosphate (PBS) buffer rinses. The attached cells were fixed with 2% glutaraldehyde, rinsed with PBS, then postfixed in 1% sodium-cacodylate buffered osmium tetroxide (OsO_4). Subsequently, they were dehydrated through a series of alcohol and finally embedded in situ by covering with a layer of Spurr (Polysciences Inc., Warrington, Pennsylvania, USA) resin. This was allowed to polymerize. The Spurr complex, containing the cells was then separated from the

underlying Ti-disc by partial immersion in liquid nitrogen. After contra-embedding of the specimens, ultrathin sections were cut on an ultra microtome (Reichert-Jung OMU-3) with a diamond knife (Drukker International, Cuijck, The Netherlands). These were contrasted with uranyl acetate and lead citrate. The sections were examined in a Jeol 1200 EX III transmission electron microscope (TEM).

In addition to TEM, X-ray microanalysis (EDX) was performed to obtain information about the composition of the produced ECM and cellular accretions. In the specimens for this part of this study, post fixation by OsO_4 was omitted, to prevent from overlap of signalled Osmium peaks. Unstained ultra thin (purple to brownish zone) sections were used for single spot or line EDX with a NORAN Instruments TN-5502 NE x-ray microanalyser. Substantial thickness of the section is required to detect elements of calcium and phosphorous. Spot measurements were performed during 100 seconds lifetime.

In the TEM and EDX study, only the four experimental materials were used. No reference material was included, for technical reasons at preparing ultra thin sections.

7.3 RESULTS

7.3.1 Characterization of the Ca-P coatings

SEM examination revealed that the 3 different sputter conditions resulted in a uniform surface covering of the titanium substrates. Further, all coatings exposed the presence of very small surface pits (diameter < 500 nm) (Figure 1).

XRD demonstrated that all coatings had an amorphous structure and RBS showed that the coatings had the following Ca/P ratio's:

* CaP400 Ca/P=2.03 +/- 0.04
* CaP400O2 Ca/P=1.77 +/- 0.02
* CaP400H2O Ca/P=1.79 +/- 0.02.

FTIR measurements showed for all coatings two clusters of P-O peaks from 900-1150 and 550-600 cm^{-1}. OH bonds were not detected, all coatings had a wide peak over the region from 2800 to 4000 cm^{-1} indicative for water absorption.

7.3.2 Cell Proliferation

The proliferation data of the RBM cells on the different substrates are presented in Figure 2.

Incident light inspection confirmed the complete removal of the cells from all surfaces after trypsinization. Statistical testing of the findings, using a two-way analysis of variance (ANOVA) and a multiple comparison test (Tukey), revealed

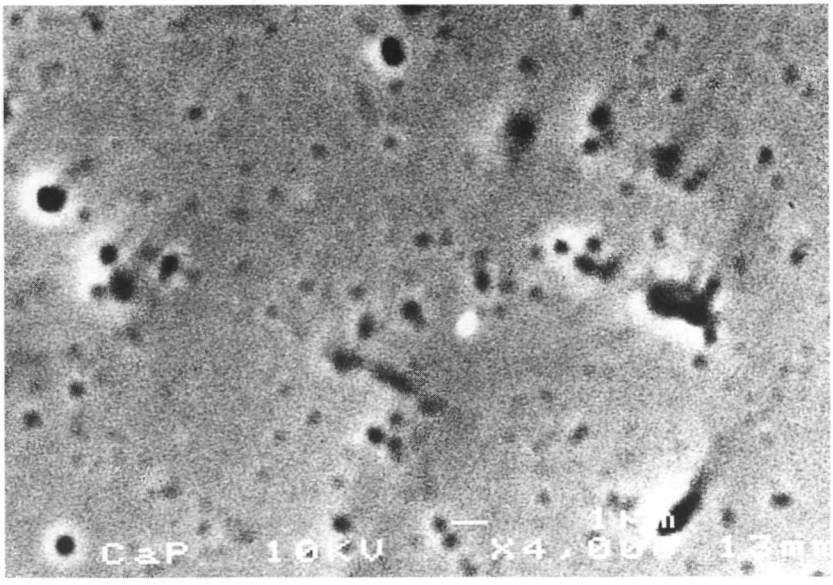

Figure 1 Scanning micrograph of an as sputtered CaP400 coating. Small surface pits (diameter < 500 nm) are shown.

that the proliferation rates were significantly higher for cpTi and Thermanox than for all RF magnetron sputter coated substrates ($p<0.05$).

7.3.3 Extracellular Matrix Production

Figure 3 shows the results of the tetracycline labelling experiments. Figure 4 demonstrates that adequate fluorescence was obtained with CLSM. Two-way analysis of variance (ANOVA) and a multiple comparison test (Tukey) revealed a significant difference in calcified mineralized matrix production between Ca-P sputter-coated and non-coated specimens ($P<0.05$). Further, the differences found between the various Ca-P coatings were also significant ($P<0.05$).

7.3.4 Coating Dissolution

After 3 days of incubation some coating dissolution was observed by SEM. At this time point, no difference in surface morphology between CaP400 and CaP400O2 was found. In contrast, the surface appearance of the CaP400H2O coating was more smooth. For all coatings the degradation was not increased after 5 days.

Figure 5 shows the RBS spectra of the tested coatings. It can be seen that the surface of all coatings shows signs of detoriation, characterized by the loss of Ca^{2+}-ions. No difference was observed between 3 and 5 days of incubation. Ca/P ratio calculations indicate for:

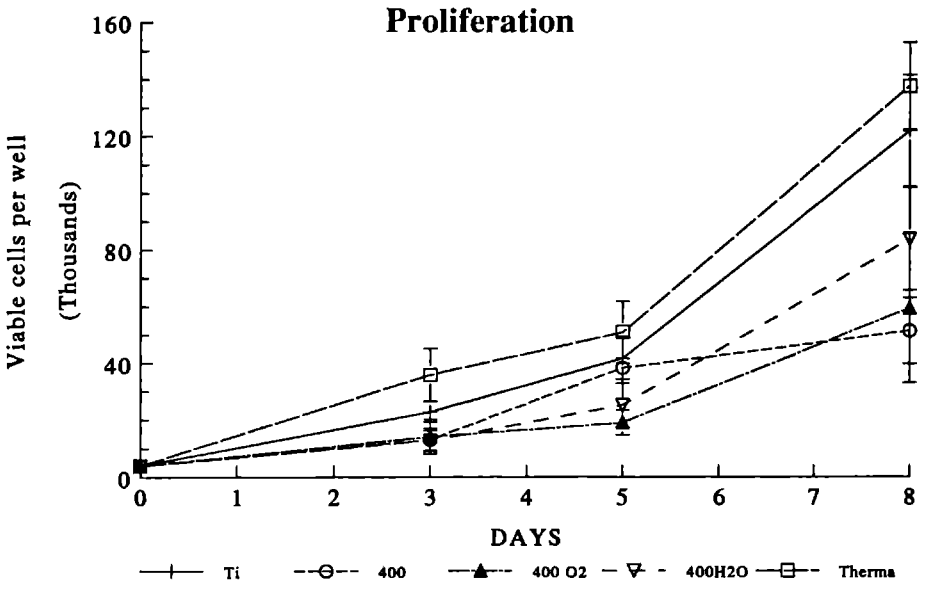

Figure 2 Diagram presenting RBM cell proliferation at 3, 5 and 8 days.

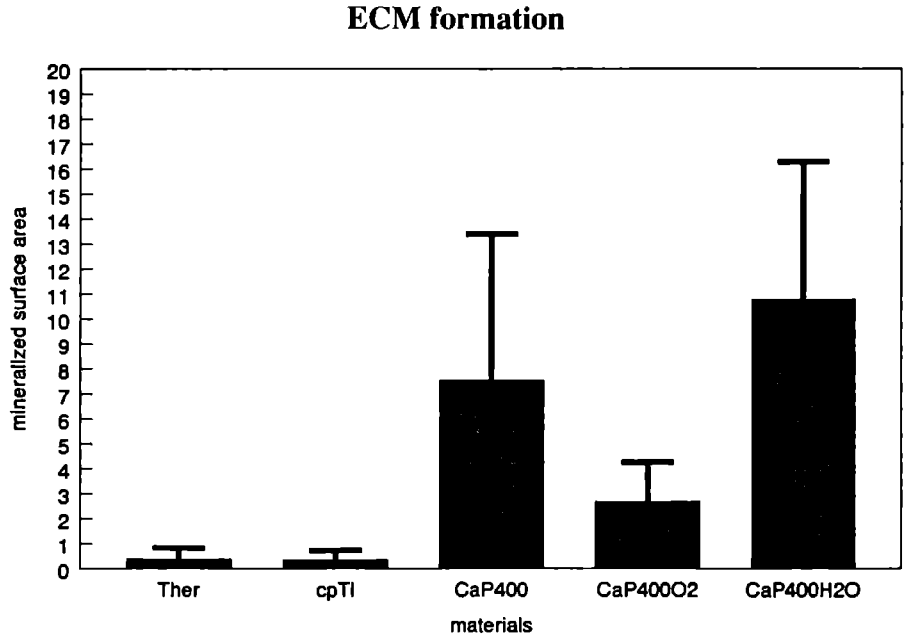

Figure 3 Bar diagram showing the results of the ECM formation. Mineralized surface area in $mm^2 \times 10^{-3}$).

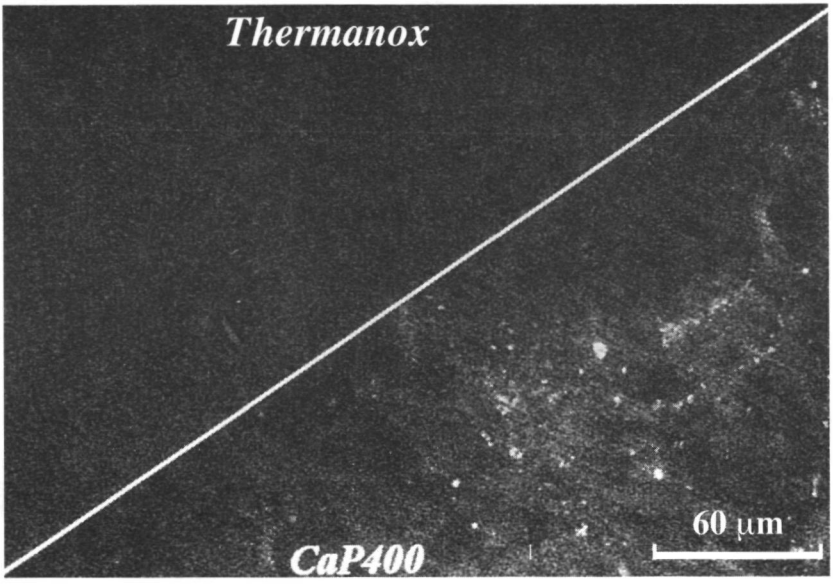

Figure 4 CLSM picture of the tetracycline labelled matrix.

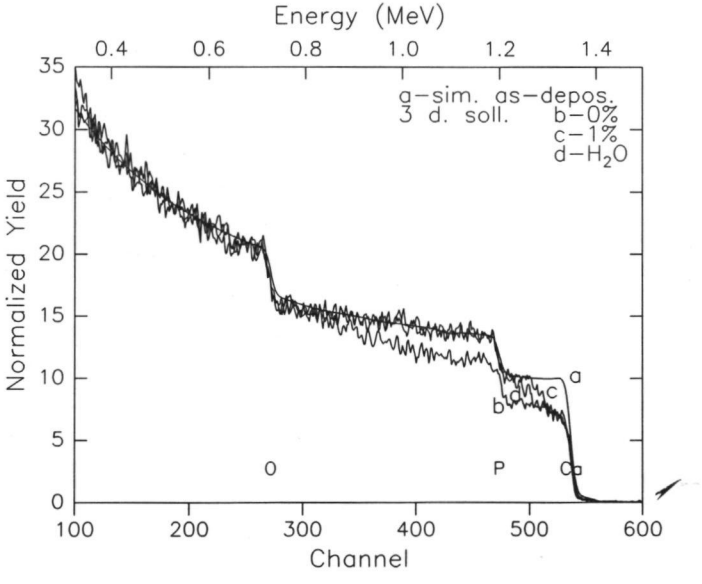

Figure 5 RBS diagram showing obviously the dissolution of Ca^{2+}-ions from the coated substrates after incubation in completed culture medium for 3 days.

1. CaP400-coatings a surface Ca/P ratio of 1.32 ± 0.1. This Ca/P ratio maintains constant over a region of 363 nm. Then it increases over a region of 332 nm to the original value of 2.03. Consequently, the total "dissolution" depth is about 700 nm.
2. CaP400O2-coatings a surface Ca/P ratio of 0.78 ± 0.15. This ratio maintains constant over a region of 4 nm and increases then over 100 nm to the original bulk value of 1.77. Total "dissolution" depth is about 100 nm.
3. CaP400H2O-coatings a surface Ca/P ratio of 1.07 ± 0.15. This ratio is constant over 10 nm and increases again over a region of 125 nm to the original bulk value of 1.79. Total "dissolution" depth is about 135 nm.

Considering the FTIR spectra, incubation in medium changed the appearance of the PO-bonds compared with the starting material. Further, all coatings revealed additional amide-peaks between 1425 and 1650 cm^{-1}, suggestive for protein adsorption (Figure 6).

7.3.5 Cell Morphology

SEM examination showed that all tested materials were covered with a multi layer of osteoblast-like cells after 8 days of culture, similar as described before (Hulshoff, 1995). To examine the RBM cell-substrate interface more closely, a scratch was created through the cell layers using a sharp knife. This revealed that on all surfaces no formation of bone-like tissue was observed. In addition, no collagen fibres could be identified (Figure 7).

Close inspection of the coatings revealed that, at 8 days, substantial thickness of the sputtered films was maintained. Still, all coatings showed some signs of superficial degradation. The appearance of the surface damage was different for the three coatings. The CaP400 surface looked very similar to the as-deposited surface. In contrast, the CaP400O2 and CaP400H2O surface showed an increase in small resp. big pit-like surface defects (Figure 8).

At 16 days of culture, clear differences were observed between the non-coated and coated specimens. SEM showed that cells cultured on cpTi substrates had formed a thin layer of calcified globular accretions (Figure 9). Only a very limited amount of collagen bundles associated with this underlying cement-like structure could be distincted. Ca-P coated substrates also supported the formation of mineralized globuli. On the other hand, compared with cpTi-discs, the deposited layer was significantly thicker and was associated with a rich fibrous collagen matrix (Figure 10). In between the collagen fibers abundant production of globular accretions was seen. Although, all coatings were clearly degraded, there were differences in the amount of degradation. The CaP400O2 and CaP400H2O layers

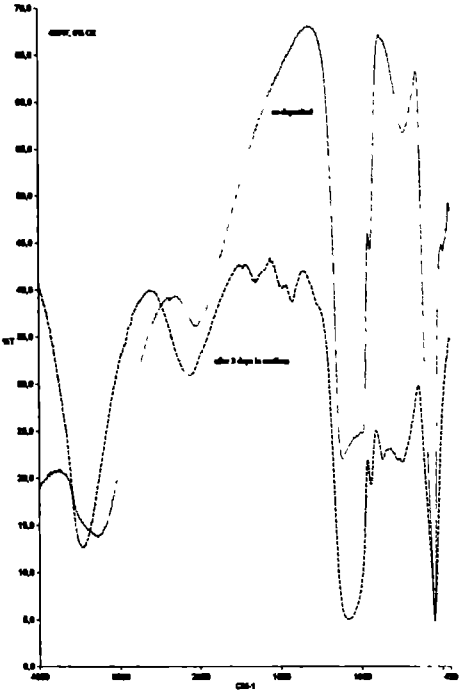

Figure 6 FTIR spectra of a CaP400 coated substrate before and after 3 days in completed culture medium showing addional amide peaks between 1425 and 1650 cm^{-1}.

were almost completely disappeared (Figure 11). Only, locally some remnants were maintained. While of the CaP400, a substantial part of the coating was still present (Figure 10A). The small cracks, that can be seen in the maintained coating parts, are a result of the SEM preparation process.

TEM examination confirmed these SEM findings. At 16 days, multi layers of osteoblast-like cells were evident over all the substrate surfaces (Figure 12A). The cells were elongated. No difference in cell spreading behaviour on titanium and Ca-P surfaces could be observed. Prominent structures within the cells were nucleus, mitochondria, ribosomes and rough endoplasmatic reticulum. In the cell layer, which was closest to the substrate surface, more endoplasmatic reticulum was seen than in the superficial cell layers. Inside the cells also numerous mineralized bodies were seen. Further observation revealed that the plasma membrane was lined with coated and non-coated endocytotic structures (12 B and C). These were present on the cell surface facing the substratum as well as on the cell surface exposed to the culture medium.

Despite this similarity in general appearance of the cell cultures on the different materials, a clear difference was observed in the formation of collagen bundles and

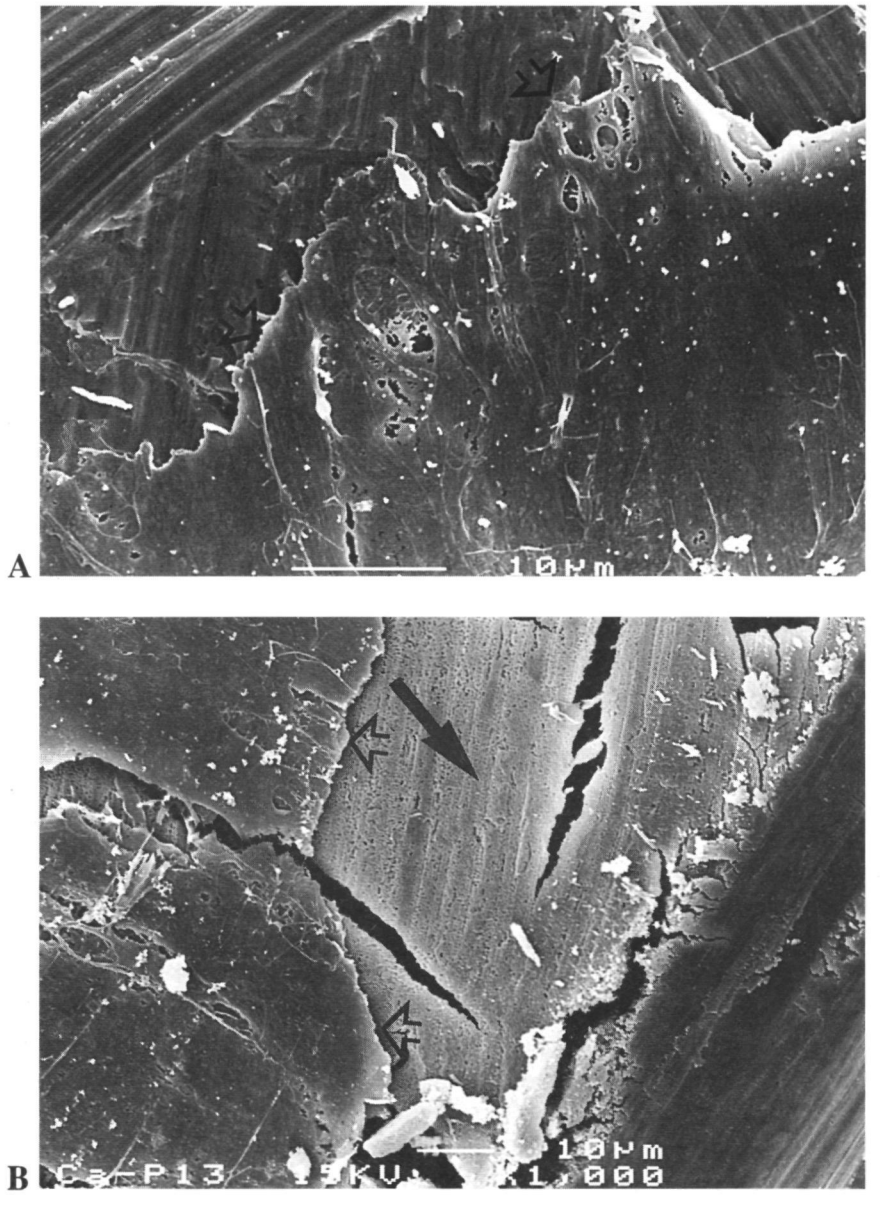

Figure 7 Scanning micrographs showing a multilayer of RBM cells (open arrows) cultured for 8 days on,
A. cpTi
B. CaP400O2.
On both specimens, no signs of mineralization can be recognized. Further, the coating surface (arrow) shows only slight signs of detoriation.

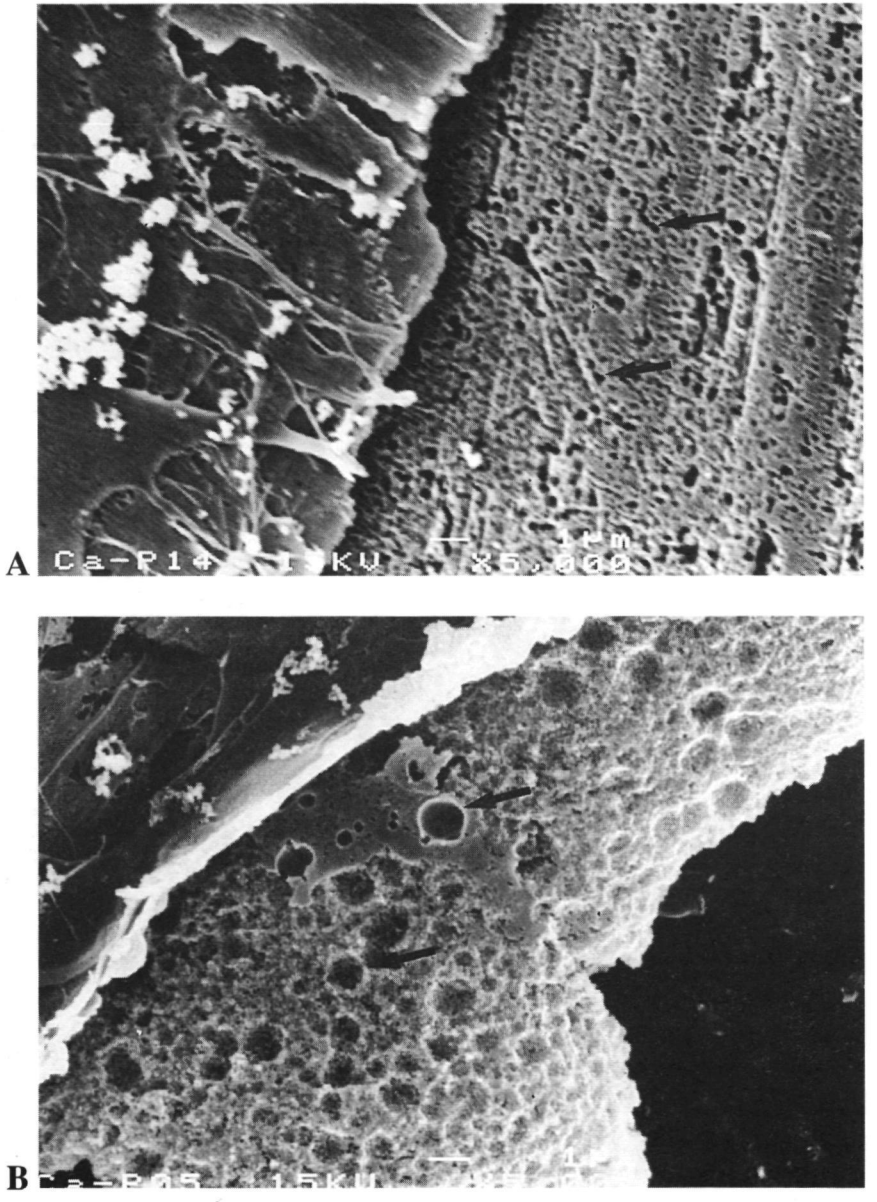

Figure 8 CaP400O2 (A) and CaP400H2O (B) substrate after 8 days of cell culture. Signs of superficial degraation are visible as characterized by the presence of small and big surface pits (arrows).

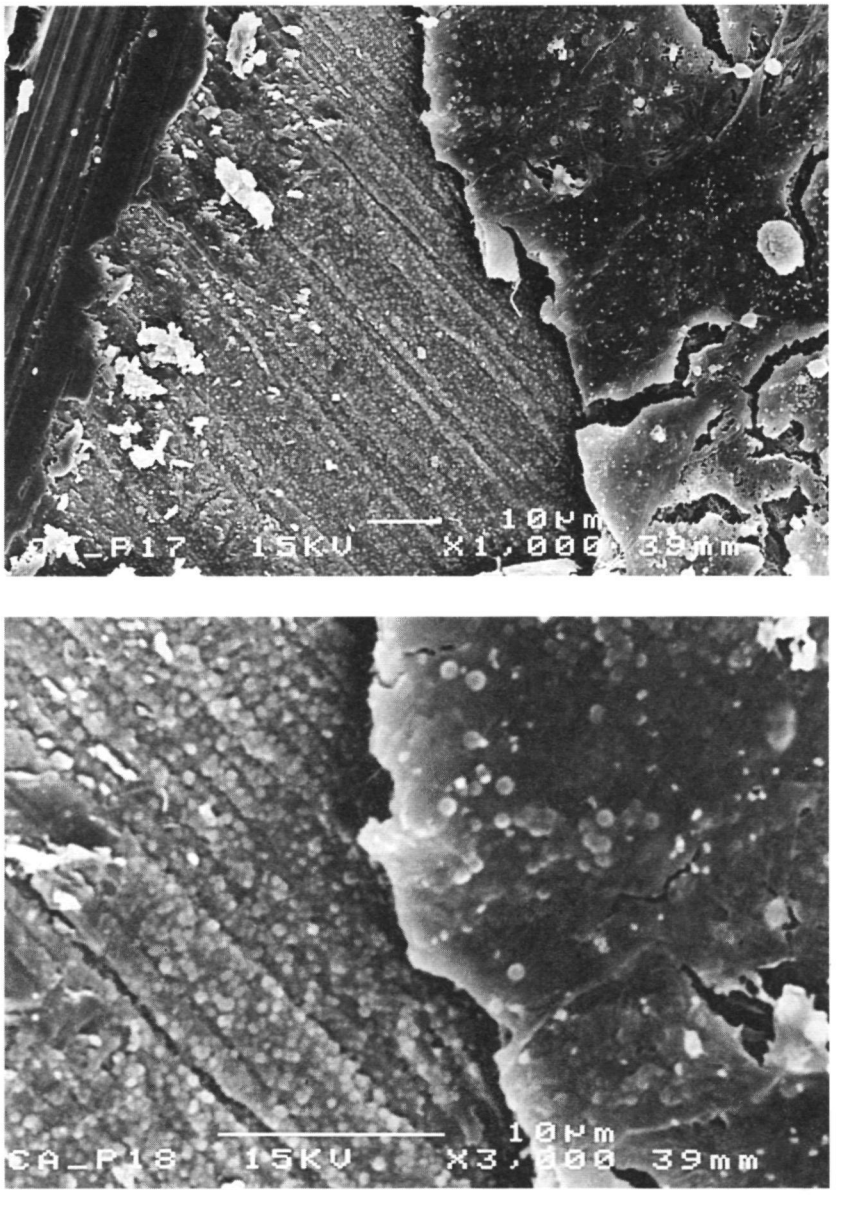

Figure 9 After 16 days of culture, on cpTi only a thin layer of calcified globular accretions is found (figure B shows a detail of A).

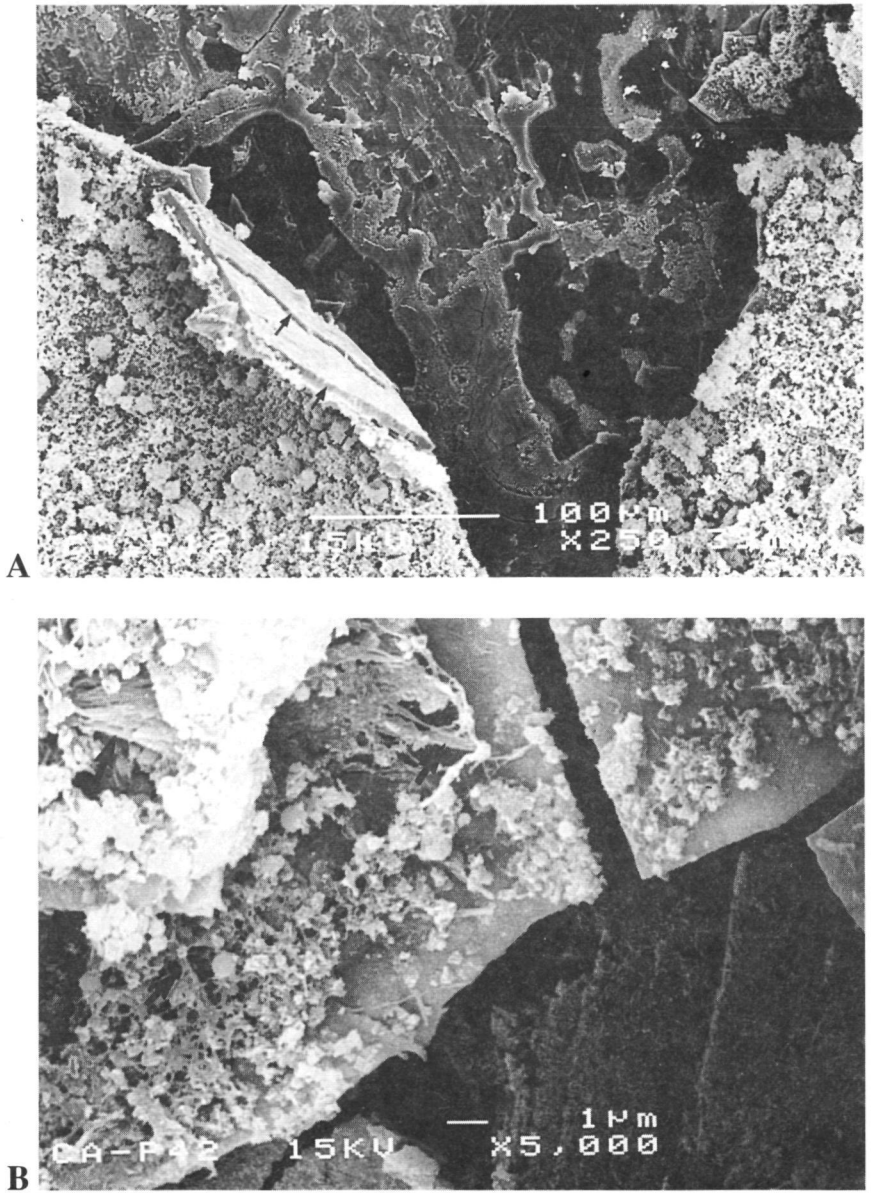

Figure 10 SEM pictures showing a CaP400 and CaP400H2O substrate after 16 days of incubation. On both surfaces, besides globular accretions, a rich fibrous collagen matrix is associated with the osteoblast like cells.

A. CaP400 substrate: it can be seen that still a substantial part of the coatings is still present. Where the cell layer is lifted from the surface (arrows), three distinct layers can be observed, representing the thin coating layer, a thick ECM and a multilayer of cells.

B. CaP400H2O substrate: an abundance of ECM with collagen fibres (arrows) can be observed.

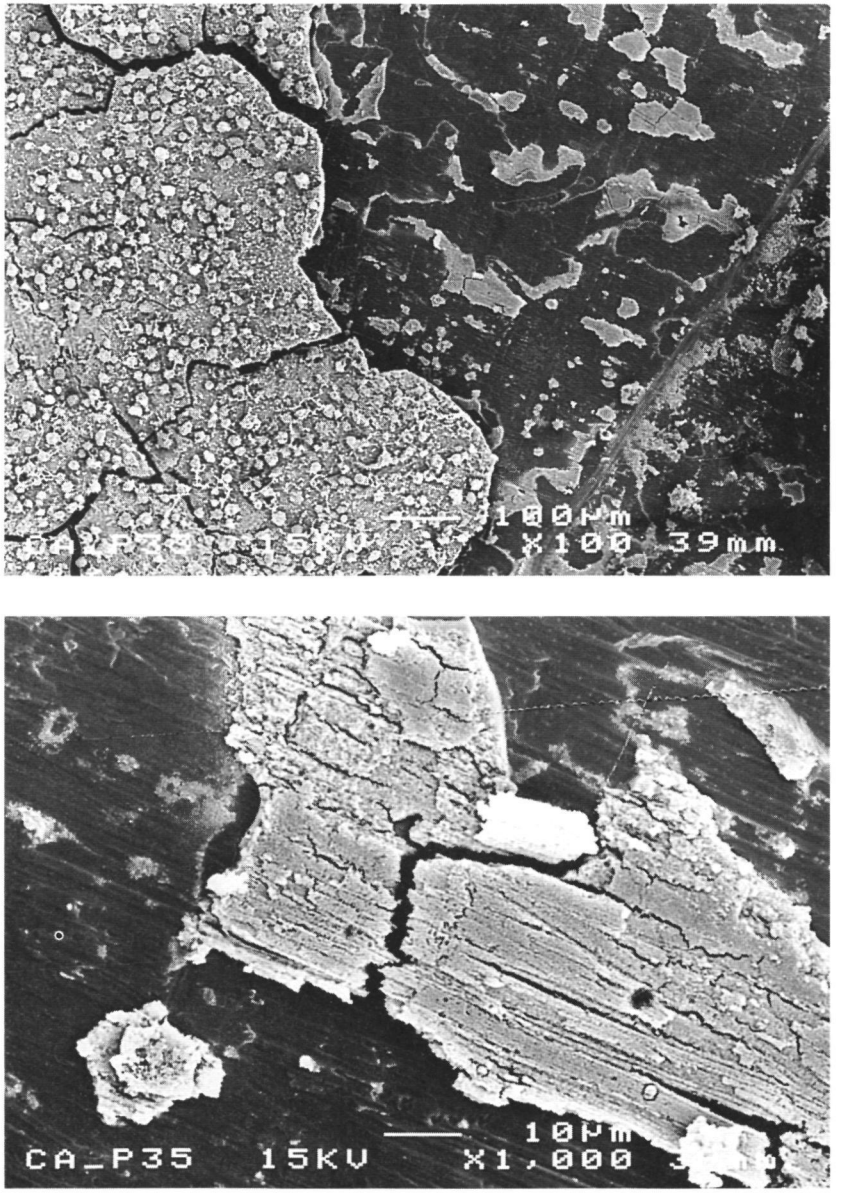

Figure 11 Scanning electron micrographs of RBM cells cultured on CaP400O2 for 16 days.
A. A multilayer of RBM cells with its produced ECM is shown. Just remnants of the coating are left.
B. Represents a detail of this partially degradated coating.

bone matrix. Figure 13 shows a representative example of the TEM appearance of the cpTi-interface. The substrate surface is covered with a thin cement-like structure. On Ca-P coatings the interface showed two appearances (Figure 14 and 15). As illustrated in Figure 14A, in areas where the original coating was maintained, an additional layer of about 1-3 μm thickness was deposited. This layer could be easily discerned, because it was less dense. The top of this less dense layer was covered with a dense line of approximately 500 nm thickness. Small needle-like foci of mineralization (Figure 14B) were observed at this layer. Collagen fibres were present between cells and substrate surface as well as between individual cells. At places were the coating was disappeared (Figure 15), coating degradation was followed by the deposition of a dense mineralized matrix of about 2 μm thickness. Cells were in close contact with this interfacial layer.

7.3.6 Energy Dispersive X-ray Microanalysis (EDX)

EDX confirmed the presence of Ca and P in the dense accretions as observed inside the cytoplasm of the RBM cells. Further, Ca and P were detected in the coating, as left after using the fracture technique, and in the ECM deposited on the coating. The Spurr resin, used for embedding of the specimens served as a negative control. Ca and P peaks could not be observed at locations where only Spurr was present. No quantitative data about the Ca/P ratio of the measured positive areas could be obtained.

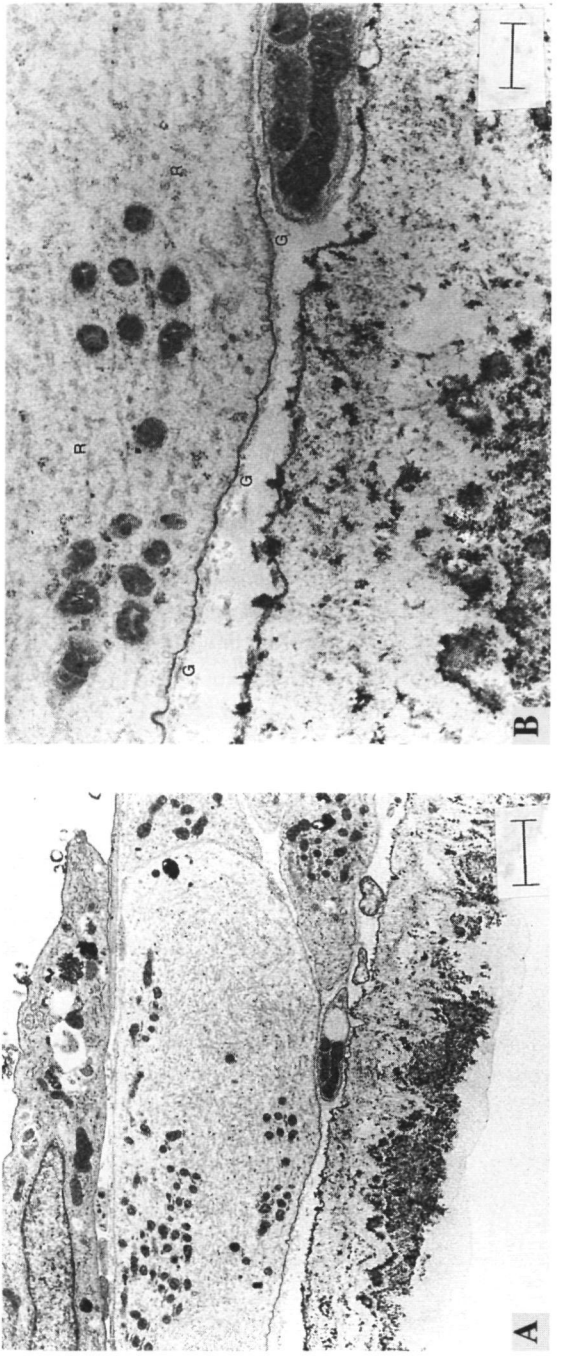

Figure 12 TEM picture of RBM cels cultured for 16 days on a CaP400O2 surface:
A. Overview, bar = 1.54 µm.
B. Detail of growing crystals, bar = 400 nm.
C. Detail showing calcified body, bar = 650 nm.
M = mitochondria;
R = areas of ribosomes;
Ca = calcified body;
G = collagen.

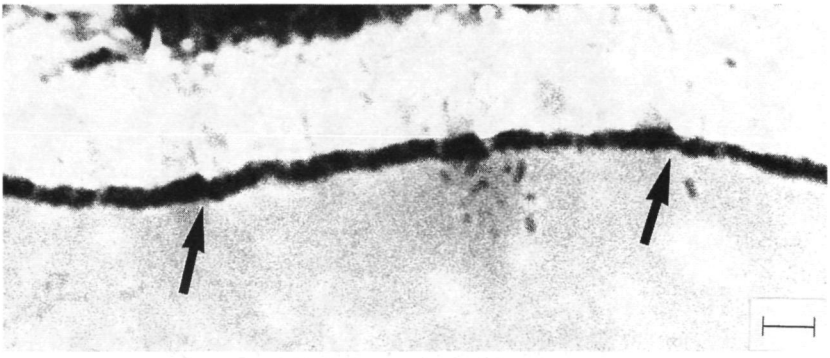

Figure 13 Transmission electron micrograph of the cpTi interface. A thin cement-like line structure (arrows) is interposed between cells and titanium substrate. (bar = 208 nm)

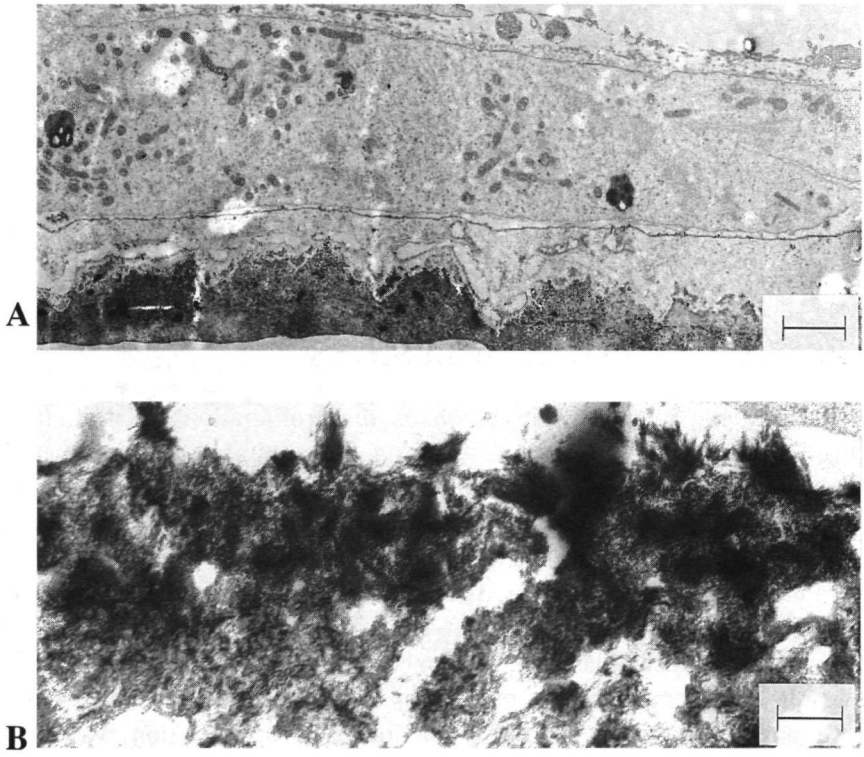

Figure 14 TEM picture of RBM cells cultured for 16 days on CaP400O2 showing the appearence when the coating is maintained:
A. Overview, bar = 1.31 μm.
B. A detail of the growing needle-like foci of mineralization, bar=340 nm.

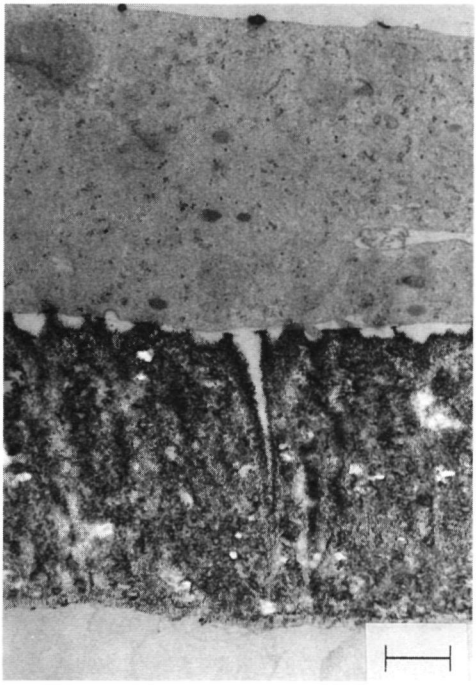

Figure 15 TEM picture of cells cultured for 16 days on CaP400H2O. A dense mineralized matrix has been deposited. An osteoblast-like cell is in close contact with this interfacial layer. (bar = 500 nm)

7.4 DISCUSSION AND CONCLUSIONS

In this study we obtained information on the proliferation and formation of mineralized matrix by rat bone marrow stromal cells cultured on Ca-P materials. The cellular morphology and cell/ substrate interface was studied for different RF magnetron sputter Ca-P coated substrates and compared to uncoated titanium. The results demonstrated that bone formation proceeded faster on the used Ca-P surfaces. Further, this effect appeared to be dependent of the Ca-P ratio of the deposited coatings. With this study we are the first to present TEM results of Ca-P coating, interface and osteoblast-like cells in one and the same ultrathin section.

The results complement one of our earlier *in vitro* studies (Hulshoff, 1995) in which we analysed only RBM cell behaviour and no attention was paid to compositional changes both in the coating as well as interfacial layer. Some of the current observations need additional explanation.

First, we noticed that the degradation of the Ca-P sputter coatings was associated with a change in Ca/P ratio of the superficial coating layer. Apparently,

the depth and measure of this decrease was dependent of the used sputter parameters. Ca/P values between 0.8 and 1.3 were reached. On basis of the final loss of Ca^{2+}-ions, the surface Ca-P compound formed has to be defined as brushite/monetite (MCP) and octacalciumphosphate (OCP). This finding corroborates with the current theories about the biological sequence of bone formation. Also during the various stages in the development of hard bone tissue a wide variety of calcium phosphates is known to be present, but starting with MCP and OCP (Ravaglioli, 1992). Unfortunately, the used EDX technique appeared to be unsuited to obtain additional quantitative data about the composition of the coating and deposited ECM after incubation with cells.

The FTIR spectra showed that during incubation also proteins precipitated on the Ca-P surfaces. Although, we were unable to confirm the exact nature of this deposit, we know that proteins can have an important function in the mineralization process of mineralized tissues. They are suggested to be involved either as nucleator or inhibitor of hydroxyapatite formation (McKee, 1993,1996; Martin, 1994; Hunter, 1996). Nevertheless, notwithstanding of the exact role of these proteins, we can hypothesize that the observed increase in ECM formation on our Ca-P coatings is the result of an interfacial dissolution (Ca^{2+}-ions) and adsorption/precipitation (proteins and apatite) process. Further, we have to emphasize that also the original substrate properties and deposited ECM can already have a pronounced influence on the differentiation and expression of bone marrow stromal cells. For example, Healy (1996) and Ozawa (1996) proved that surface chemistry of the substrate material can increase the rate of matrix mineralization of cultured marrow cells. While Hasegawa (1994) demonstrated by morphological and biochemical studies that the nature and conformational state of the offered proteinaceous matrix are important for nodule production and osteocalcin secretion of osteoblast-like cells. It can be supposed that the difference in ECM formation on the various Ca-P sputter coatings is due to variations in the adsorbed proteinaceous layer. More research has to be performed to confirm this theory.

A second point that needs further explanation is the observed degradation of the experimental coatings. During the last years, the final aim for the application of Ca-P coatings on medical implants has been a frequent topic of discussion. A lot of arguments have been pleaded both opposite and in favour for the use of coatings (Geesink, 1993). This debate was fed by clinical implant failures supposed to be due to coating resorption or delamination (Kay, 1992; Bloebaum, 1994; Wheeler 1996). In this scope, to our opinion, the only reason for the use of Ca-P coating is to improve bone formation during the initial healing event. Thereafter, the coating has to disappear. Consequently, we do not consider the occasionally fast degradation of the used amorphous-sputter coatings as a problem. Certainly,

because as shown in the SEM and TEM assays, these coatings fulfilled their biological role by enhancing mineralized matrix formation. Besides, the observed difference in degradation time and ECM formation between the various coatings, suggests that by changing the sputter parameters, the final coating response can be modelled.

SEM experiments also showed that the produced ECM could be easily separated from the underlying substrate surface. As confirmed by Hanawa (1992), a chemical bond can develop between calcium phosphates and titanium. This occurs due to incorporation of calcium and phosphorus in the surface oxide layer. However, *in vivo* experiments revealed that this formation mechanism is a slow process which takes considerable time before a sufficient adhesion strength is obtained (de Groot, 1994). The weak adhesion of this layer also corroborates with experiments (Tanahashi, 1995a,1995b; Hata, 1995; Nagano, 1996), in which bone-like apatite layers are grown on various materials, including polymers and stainless steel, by soaking in simulated body fluid (SBF). Although significant thick layers are formed already within two weeks, the adhesive strength of the deposited biological apatite is very low. Nevertheless, the clinical consequences for the initial poor mechanical properties of the ECM on our Ca-P sputter coatings are probably not so relevant. Especially, since: (1) the adhesion strength of the as-sputtered films is very high, so that no delamination problems will occur during implant installment, and (2) it can be supposed that, around the currently used non-coated titanium implants, at best similar phenomena will develop at a reduced ECM formation rate.

Finally, a remark has to be made about the extrapolation of the *in vitro* results to the *in vivo* application. The osteogenic phenotype of bone marrow cells is determined by the concentration of dexamethasone and ß-glycerophosphate added to the culture medium (Maniatopoulos, 1988; Hasegawa, 1994; Cheng, 1996; Davies, 1996; Taira, 1995). The function of the glucocorticoid is to stimulate the alkaline phosphatase (ALP) expression of the cultured bone marrow cells. Subsequently, the produced ALP hydrolyses the ß-glycerophosphate. This induces an increase in local phosphatase concentration leading to mineralization of the deposited collagen matrix. The question which then arises is whether in the local micro environment around an implant sufficient concentrations of organic phosphate are available to cause the same effect. This also in relation to the observed degradation of the used amorphous coatings. Unfortunately, this question cannot be answered yet.

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CHAPTER 8

FINAL REMARKS

As demonstrated, the application of RF magnetron sputtering for the deposition of Ca-P ceramic coatings on oral implants is a promising technique. In this chapter the most important results as obtained will be discussed. Finally, recommendations for future research will be made.

8.1 RETROSPECTIVE

Biomaterials are used to direct, supplement, or replace the function of living tissues. Currently, there is an increasing need for the development of biomaterials eleciting a predictable and controlled response. In addition to surgical and biomechanical factors, we know that the bone formation and regeneration process around an implant is influenced by the physico-chemical surface properties of the applied biomaterial. For this reason, Ca-P ceramic is preferred as bone substitute, since this bioactive material is supposed to favor the realisation of the required harmonious interface. On the other hand, it has also been noticed that the fatigue properties of bulk Ca-P ceramics are insufficient for load bearing applications, like oral implants.

These considerations formed the argument for the study, as described in this thesis. We focussed on the biological testing of an experimental Ca-P coating, which is supposed to be superior in terms of resorption and delamination above plasma-sprayed Ca-P coatings.

The cell culture studies have established that the RF magnetron sputtered Ca-P coatings indeed possess the capacity to activate the differentiation and expression of osteogenic cells. Besides, we confirmed again that the bone formation process proceeds faster on Ca-P surfaces than on commercially pure titanium. This osteogenic capacity of the experimental coatings was also observed in most of our animal experiments, where oral implants were installed in the high density trabecular bone of the femoral condyle.

Unfortunately, these results could not be confirmed for sputter coated implants placed in the low density bone of the goat maxilla. It can be supposed that the overall bone healing response is delayed in this type of bone. Then it is possible, that the experimental sputter coatings dissolve too fast, before they even had the chance to influence the bone response. This hypothesis is supported by:

1. percentages of bone contact as measured on the implants of the torque study in chapter 5

2. the observed dissolution of similar amorphous coatings in the cell culture experiments in chapter 2 and 7.

Although, these findings can also suggest that sputter coatings suffer from the same dissolution problem as plasma-sprayed coatings, this conclusion is not justified. First, because the only purpose of a Ca-P coating is to enhance the initial bone healing. After this function is fulfilled the coating can or perhaps has to disappear. Second, degradation of plasma-sprayed coatings is frequently associated with fragmentation and loosening of Ca-P particles, which can result in an osteolytic bone response. The resorption of sputter coatings proceeds in a more uniform way. Apparently, this is due to the completely different deposition process; atoms or molecules in RF magnetron sputtering vs. powder particles in plasma-spraying.

Perhaps, the most important observation presented here was that the bone formation effect appears to be dependent of the Ca/P ratio of the used Ca-P coating. This finding supports the hypothesis that the bioactive properties of Ca-P ceramic can be specifically designed to deliver medical and oral implants with a predictable bone response.

8.2 PROSPECTIVES

The results of this study lead to the following recommendations for future research:

- Knowledge of the parameters which control the cascade of events, involved in the bone response to Ca-P biomaterials, is a prerequisite to produce implants with the required biological properties. Therefore, the response of bone cells at the biointerface should be examined both from a cell biological as well as physicochemical point of view. To achieve this, a strong relationship between engineering and life sciences has to be established (Nerem, 1995). Expertise at the level of biomaterials, cell biology and biophysics is required to carry out such a study.
- The successful remodeling of the bone around oral implants has to be considered as the final outcome of a sum in which several variables are involved. In this context, in different studies, (Wennerberg 1995, 1996; Buser 1991; Gotfredsen 1995) the advantages of a roughened oral implant hase been observed. Consequently, it can be questioned whether bone healing can be further improved by the combination of a roughened surface with a Ca-P sputter coating. At the moment, this research appears to be very relevant, since we know that a thin sputter coating almost does not change the original surface roughness of the implant.
- As all our results are obtained in more or less non-loaded experimental models and at relatively short implantation periods, the clinical relevance of our findings is not demonstrated yet. Although, bone formation around non-coated

titanium implants only seems to be delayed and finally achieves a similar fixation (de Groot, 1994), it still has to be proven that the same is true for gradually resorbed or remodeled Ca-P coatings. Therefore, long-term studies on the final prognosis of coated implants have to be performed.

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CHAPTER 9

SUMMARY

SUMMARY

The presence and maintenance of the proper bone response is an important condition for the clinical success of an oral implant. Therefore direct bone-implant contact without an intervening connective tissue layer or even direct bone bonding is required. The used implant material is a factor of influence to obtain the above mentioned prefered situation. Hydroxyapatite ceramic (HA) is a material with excellent biological properties. Unfortunately, it is too brittle and has poor fatigue properties in loaded applications. Therefore calciumphosphate ceramic (Ca-P), like HA, can only be applied as a coating on mechanically strong titanium oral implants. The primary aim of this thesis was to evaluate the biological characteristics and suitability of experimental radio frequency (RF) magnetron sputtered Ca-P caotings for oral implants.

In **Chapter 2** a rat bone marrow cell culture was used to evaluate the osteogenic potential of amorphous and crystalline RF magnetron sputter Ca-P coatings. The coatings were deposited on titanium discs. Amorphous and crystalline plasma spray Ca-P coated and non coated titanium discs served as reference material. The cellular behaviour was analyzed with quantitative (attachment and proliferation rates) and qualitative (scanning electron microscopy) techniques. No significant differences were found in cell attachment and proliferation rates between the various materials. Scanning electron microscopy showed extracellular matrix formation after 18 days of culture on amorphous plasma-sprayed and the two types of magnetron sputtered coatings. Furthermore, no severe degradation of the magnetron sputtered coatings was observed. They even appeared to induce apatite formation.

Chapter 3 describes the bone response to different plasma spray and RF magnetron sputter Ca-P coated implants. Four types of Ca-P coatings were investigated; a plasma spray Ca-P coating (HA-PS), a heat treated plasma-spray Ca-P coating (HA-PS/ht), an amorphous magnetron sputter coating (Ca-P-a) and a crystalline magnetron sputter coating (Ca-P-c). Implants were inserted in the lateral and medial femoral condyles of 18 rabbits. After implantation periods of 3, 6 and 9 weeks, the bone implant interface was evaluated histologically and histomorphometrically. Bone contact and the amount of bone surrounding the implant-bone interface was measured. Microscopical examination revealed that all types of coatings followed the same process of bone healing. Measurements of bone contact at 6 and 9 weeks did not reveal significant differences between the various coatings. For the amount of bone, in a circular region at a certain distance from the implant, the Ca-P-c coated implants showed a significantly greater

amount of bone after 6 weeks of implantation, than the other three Ca-P coatings. At 9 weeks this difference could no longer be measured.

On basis of these findings we concluded that magnetron sputtered Ca-P coatings show the same process of bone healing as the plasma sprayed Ca-P coatings when inserted into the trabecular femoral bone of rabbits.

Following these favorable results, in **Chapter 4,** the bone reponse to different Ca-P coated implants was evaluated in the low density bone of the goat maxilla. Two types of plasma spray coatings were applied to a commercially pure titanium (cpTi) tapered, conical screw design implant (BioComp®); hydroxyapatite (HA-PS) and a dual coating, consisting of FA and HA (FA/HA-PS). In addition an amorphous RF magnetron sputter coating (Ca-P-a) and non-coated implants were investigated. Implants were inserted in the maxilla of adult female goats. After implantation periods of 3 and 6 months, the bone implant interface was evaluated histologically and histomorphometrically. After both implantation periods all plasma spray coated implants were maintained. On the other hand 3 Ca-P-a and 2 cpTi implants were lost. Histological examination revealed a better bone response to both plasma spray coated implants. Histomorphometrical evaluation confirmed this finding. At 3 and 6 months significantly higher percentages of bone contact were measured for both plasma spray coated implants than for the cpTi and Ca-P-a implants, while no significant difference existed between both implantation periods. Degradation of both plasma spray coatings was observed. Supported by the results we concluded that, although Ca-P coatings can improve the performance of dental implants, the presence of a Ca-P coating is not the only important factor for bone healing around implants placed in low density trabecular bone. These results were suggestive for the influence of surface roughness to obtain successfull remodeling around an oral implant.

Therefore in **Chapter 5** the bone response to Ca-P plasma-spray and RF magnetron sputter coated implants with comparable roughness was investigated. Again, tapered conical screw designed implants were installed in the trabecular bone of the femurs of 9 goats. They were provided with 2 types of coatings, the plasma spray dual coating of fluorapatite and hydroxyapatite (FA/HA-PS) and a titanium plasma spray coating, covered with an amorphous Ca-P magnetron sputtercoating (TPS/Ca-P-a). These implants were evaluated histologically and mechanically after 3 months of implantation. A well controlled method to apply and measure a torsional force to load the screw type implants to the point of failure was introduced.

All implants healed uneventful and were well fixed. No significant difference for the torsional failure force was measured for both types of coatings.

Nevertheless, SEM revealed differently situated fracture planes. Light microscopy showed intimate bone-implant contact for both types of coatings, original drill margins were still visible. A lamellar type of bone with some remodeling lacunae was shown. Histomorphometry revealed a higher percentage of bone contact for the FA/HA-PS coated implants. Measurement of the amount of bone revealed more bone mass around TPS/Ca-P-a coated implants.

The animal model of the trabecular bone of the goat femur was used again in **Chapter 6**. Also the experimental plasma-spray bilayered Ca-P coating (FA/HA) and amorphous RF magnetron sputter coating (Ca-P-a) were evaluated. A noncoated cpTi implant served as a control. After implantation periods of 3, 12 and 24 days, light microscopical evaluation revealed that bone formation on the Ca-P coated implants proceeded faster. At 24 days higher percentages of bone contact were measured for both Ca-P coated implants than for non-coated implants. Unfortunately this difference was only significant for the FA/HA coated implants. On basis of these findings, we concluded that Ca-P coatings show improved bone response due to an initial difference in bone cell response.

Chapter 7 describes another *in vitro* study. The aim of this investigation was to obtain more understanding on the biological behaviour of these coatings by *in vitro* experiments. Therefore, the effect of non-coated titanium (Ti) and three different Ca-P sputtered surfaces on the proliferation and differentiation (morphology and matrix production) of osteoblast-like cells was studied. Proliferation was determined, and morphology of the osteoblast-like cells was studied at electron microscopical level. Besides fluorescent markers and energy dispersive X-ray microanalysis were used to obtain quantitative and compositional information about the produced calcified extracellular matrix (ECM). Results demonstrated that proliferation of the osteoblast-like cells was significantly higher on non-coated than on Ca-P coated samples. On the other hand, more mineralized ECM was formed on the coated surfaces. In addition, transmission electron microscopy confirmed that the cells on the coated substrates were surrounded by ECM with collagen fibres embedded in crystallized needle shaped structures. On basis of these findings, we conclude that: (1) the investigated Ca-P sputter coatings possess the capacity to activate the differentiation and expression of osteogenic cells, and (2) bone formation proceeds faster on Ca-P surfaces than on Ti substrates. Further, we noticed that this bone inductive effect appeared to be dependent of the Ca/P ratio of the deposited coatings.

In **Chapter 8** the final remarks and overall conclusions are made. Expectations for future research are formulated.

SAMENVATTING

De aanwezigheid en het behoud van een goede bot reactie is een belangrijke voorwaarde voor het klinisch slagen van een tandimplantaat. Daarvoor is direkt bot-implantaat contact zonder tussenliggende bindweefsellaag, of zelfs, direkte botbinding een vereiste. Het gebruikte implantaatmateriaal is één van de factoren van invloed om bovengenoemde situatie te verkrijgen. Hydroxyapatiet keramiek (HA) is een materiaal met uitstekende biologische eigenschappen. Helaas is het materiaal te breekbaar en heeft het slechte vermoeidheids eigenschappen, wanneer het wordt gebruikt in belaste toestand. Daarom kan calcium fosfaat keramiek (Ca-P), zoals HA, slechts worden toegepast als deklaag op een mechanisch sterk materiaal, zoals bijvoorbeeld titanium. Het doel van dit proefschrift is de geschiktheid van de radio frequency (RF) magnetron sputter techniek voor het vervaardigen van keramische deklagen voor tandimplantaten, en de biologische eigenschappen daarvan, te onderzoeken.

In **hoofdstuk 2** werd een ratbeenmerg celkweek techniek gebruikt om de botvormende capaciteit van amorfe en kristallijne RF magnetron gesputterde Ca-P deklagen te evalueren. De coatings werden op titanium schijfjes aangebracht. Amorfe en kristallijne plasma spray gecoate en ongecoate titanium schijfjes dienden als referentiematerialen. Het celgedrag werd met behulp van kwantitatieve (hechtings- en groeipercentages) en kwalitatieve (scanning electronen microscopie) technieken beoordeeld. Tussen de verschillende materialen onderling werden geen significante verschillen gevonden voor celhechtings- en groeipercentages. Scanning electronen microscopie toonde aan dat na 18 dagen celkweek extracellulaire matrix werd gevormd op de amorfe plasma spray coatings en op beide magnetron gesputterde coatings. Voorts werd voor de magnetron gesputterde coatings geen ernstige degradatie waargenomen. Apatiet vorming lijkt zelfs te worden veroorzaakt door deze coatings.

Hoofdstuk 3 beschrijft de botreactie ten opzichte van verschillende plasma spray en RF magnetron sputter Ca-P gecoate implantaten. Vier soorten Ca-P coatings werden onderzocht; een plasma spray Ca-P coating (HA-PS), een hitte behandelde plasma spray coating (HA-PS/ht), een amorfe magnetron sputter coating (Ca-P-a) en een kristallijne magnetron sputter coating (Ca-P-c). De implantaten werden geplaatst in de laterale en mediale condyles van de femora van 18 konijnen. Na implantatie termijnen van 3, 6 en 9 weken werd het bot-implantaat scheidingsvlak histologisch en histomorfometrisch beoordeeld. Daarvoor werd het percentage botcontact en de hoeveelheid omvattend bot rond het scheidingsvlak gemeten. Licht microscopie wees uit, dat alle soorten coatings een zelfde proces

van botgenezing met zich mee brengen. Metingen van het percentage botcontact na 6 en 9 weken lieten geen significante verschillen zien voor de verschillende implantaten. In een cirkelvormig gebied op enige afstand van het implantaat, werd na 6 weken voor de Ca-P-c gecoate implantaten een significant grotere hoeveelheid bot gemeten, dan voor de 3 andere coatings. Na 9 weken was echter niet langer sprake van een significant verschil in hoeveelheid bot.

Op basis van deze bevindingen werd geconcludeerd dat magnetron gesputterde Ca-P coatings een zelfde botgenezings reactie met zich mee brengen als plasma spray Ca-P gecoate implantaten, wanneer deze worden geimplanteerd in het trabeculaire bot van het femur van konijnen.

In opvolging van deze gunstige resultaten, werd in **hoofdstuk 4,** de botreactie ten opzichte van verschillende Ca-P gecoate implantaten onderzocht, in bot met een lage densiteit; de bovenkaak van de geit. Er werden 2 soorten plasma spray coatings aangebracht op taps toelopende, konische schroefvormige implantaten (Biocomp®); hydroxyapatiet (HA-PS) en een dubbellagige van fluorapatiet (FA) en HA coating (FA/HA-PS). Voorts werden een amorf gecoat RF magnetron sputter implantaat (Ca-P-a) en een ongecoat implantaat onderzocht. De implantaten werden geplaatst in de bovenkaak van volwassen geiten van het vrouwelijk geslacht. Na implantatie termijnen van 3 en 6 maanden, werd het scheidingsvlak bot-implantaat histologisch en histomorfometrisch geëvalueerd. Alle plasma spray gecoate implantaten werden na beide implantatie termijnen behouden. Daarentegen gingen 3 Ca-P-a en 2 cpTi implantaten verloren. Histologisch onderzoek toonde een betere botreactie ten opzichte van beide soorten plasma spray gecoate implantaten. Histomorfometrie bevestigde deze bevinding. Na 3 en 6 maanden werd een significant groter percentage botcontact gemeten voor beide plasma spray coatings, dan voor de cpTi en de Ca-P-a implantaten, terwijl voor beide implantatie periodes onderling geen significant verschil bestond. Voor beide plasma spray coatings werd degradatie waargenomen. Ondersteund door deze resultaten concludeerden we dat ofschoon Ca-P coatings de uitvoering van tandimplantaten kan verbeteren, de aanwezigheid van deze coatings niet de enige belangrijke factor is voor de botgenezing rond implantaten in trabeculair bot met een lage densiteit. Deze resultaten suggeren dat, voor het verkrijgen van succesvolle remodellerings processen rond een tandimplantaat, oppervlakte ruwheid tevens een rol speelt.

Daarom werd in **hoofdstuk 5** de botreactie ten opzichte van Ca-P plasma spray en RF magnetron sputter coatings met een vergelijkbare oppervlakte ruwheid onderzocht. Opnieuw werden taps toelopende, konische, schroefvormige implantaten geplaatst, in het trabeculaire bot van het femur van 9 geiten. De implantaten werden voorzien van 2 soorten coatings; een plasma spray dubbel

coating van fluorapatiet en hydroxyapatiet (FA/HA-PS), en een titanium plasma spray coating, die werd bedekt met een amorfe Ca-P magnetron sputter coating (TPS/Ca-P-a). Na 3 maanden implantatie werden de implantaten histolgisch en mechanisch geëvalueerd. Een goed gecontroleerde methode voor het aanbrengen en meten van een torsiekracht bij de belasting van een schroefvormig implantaat tot het punt van loskomen werd geintroduceerd.

Alle implantaten genazen zonder complicaties en zaten goed vast. Er werden geen significante verschillen gemeten voor deze benodigde torsiekracht voor de verschillende soorten coatings. Desalniettemin, toonde scanning electronen microscopie dat de breukvlakken anders waren georiënteerd. Licht microscopie toonde een goed bot-implantaat contact voor beide types coatings en voorts waren de originele begrenzingen van het boren nog zichtbaar. Een lammellair type bot met enige remodellerings lacunes was waarneembaar. Histomorfometrie toonde een groter percentage botcontact voor de FA/HA-PS gecoate implantaten. Bij het meten van de hoeveelheid bot werd meer botmassa waargenomen rond TPS/Ca-P-a gecoate implantaten.

Opnieuw werd in **hoofdstuk 6** gebruik gemaakt van het proefdiermodel van het trabeculaire bot van het femur van de geit. De dubbellagige plasma spray Ca-P coating (FA/HA-PS) en een amorfe RF magnetron sputter coating (Ca-P-a) werden geevalueerd. Een ongecoat titanium implantaat (cpTi) diende als controle. Na implantatie termijnen van 3, 12 en 24 dagen toonde lichtmicroscopie aan dat botvorming sneller plaats vond rond Ca-P gecoate implantaten. Na 24 dagen werden hogere pecentages botcontact gemeten voor beide types Ca-P gecoate implantaten, dan voor de ongecoate implantaten. Alleen voor de FA/HA-PS gecoate implantaten was dit significant verschillend. Op basis van deze bevindingen concluderen we dat Ca-P coatings een betere botreactie met zich mee brengen als gevolg van een verschil van invloed op de initiële reactie van de botcellen.

Hoofdstuk 7 beschrijft een *in vitro* experiment. Het doel van het onderzoek was meer inzicht te verkrijgen in het biologische gedrag dat deze coatings veroorzaken, door het uitvoeren van celkweek experimenten. Daarvoor werd het effect van ongecoate titanium (Ti) en 3 verschillende Ca-P magnetron sputter coatings bestudeerd, met betrekking tot de groei en differentiatie (morfologie en matrix productie) van osteoblast-achtige cellen. Proliferatie werd bepaald en de morfologie van de osteoblast-achtige cellen werd met behulp van electronen microscopie beoordeeld. Bovendien werden fluorescente markers en röntgen microanalyse gebruikt om informatie over de samenstelling en kwantiteit van de gevormde gecalcificeerde matrix te verkrijgen. De resultaten toonden dat de

proliferatie van de osteoblast-achtige cellen significant groter was op de ongecoate dan op de Ca-P gecoate exemplaren. Aan de ander kant werd op de gecoate oppervlakken meer gemineraliseerde extracellulaire matrix (ECM) gevormd. In aanvulling hierop, toonde transmissie electronen microscopie dat de cellen op de gecoate oppervlakken werden omgeven door ECM met collagene vezels welke waren geincorporeerd door kristalachtige naaldvormige structuren. Op basis van deze bevindingen werd geconcludeerd dat: (1) de onderzochte Ca-P magnetron sputter coatings de capaciteit hebben de differentiatie en expressie van botvormende cellen te activeren en (2) dat botvorming sneller vordert op Ca-P oppervlakken. Verder, werd aangetoond dat het effect van het opwekken van botvorming afhankelijk lijkt te zijn van de Ca/P ratio van de aangebrachte magnetron sputtercoating.

Tenslotte werden in **hoofdstuk 8** de laatste opmerkingen en algemene conclusies beschreven. Enkele verwachtingen voor toekomstig onderzoek werden geformuleerd.

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CURRICULUM VITAE

Anne-Gitte Hulshoff was born on March 27th, 1967 in Leeuwarden. She graduated from high school (VWO, Ubbo Emmius Lyceum at Stadskanaal) in 1985. After a one-year student exchange to Australia, she started at the Dental School Groningen in 1986. After propadeutical exams, she continued her studies at the Dental School of Nijmegen in 1987. For 4 months she studied at Århus Tandlæge Højskole in Denmark, supported by the Erasmus Exchange program in 1991. In 1992 she obtained her degree in dentistry. In the same year she started as a PhD student, on a Pioneer project supported by the Stichting voor de Technische Wetenschappen (STW), at the faculty of Medical Sciences, Department of Oral Function, Laboratory of Biomaterials of the Catholic University of Nijmegen (Head: Prof. Dr. J.A. Jansen). During this period the research presented in this thesis was carried out. Besides she was working as a dentist general practitioner in Ulft and as a prosthodontist at the Center of Special Dental Care Nijmegen.